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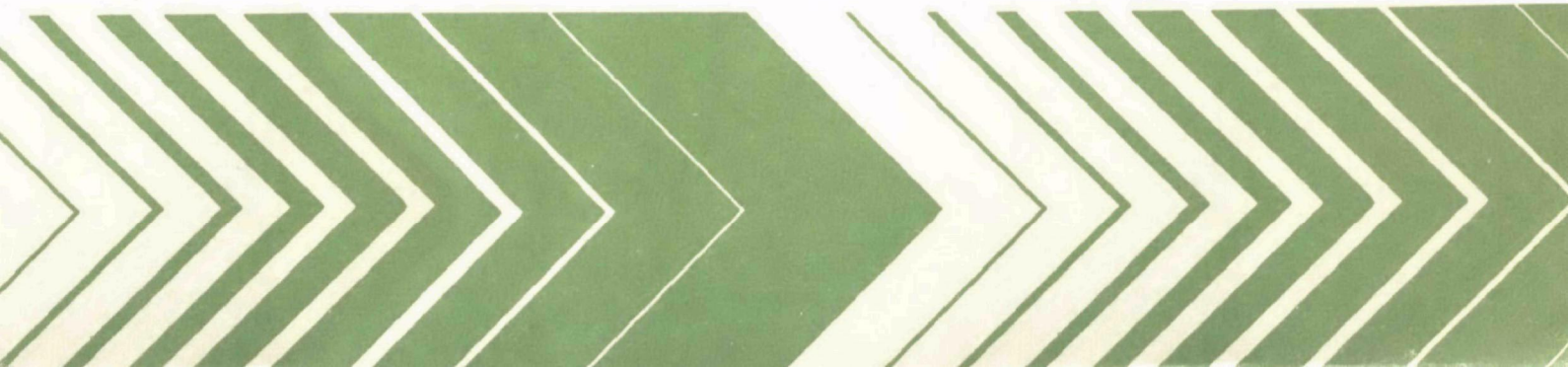
Studies of Methanogenic Bacteria in Sludge



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STUDIES OF METHANOGENIC BACTERIA IN SLUDGE

by

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Grant No. 17070-DJV

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FOREWORD

The Environmental Protection Agency was created because of increasing public and government concern about the dangers of pollution to the health and welfare of the American people. The complexity of that environment and the interplay between its components require a concentrated and integrated attack on the problem.

Research and development is that necessary first step in problem solution and it involves defining the problem, measuring its impact, and searching for solutions. The Municipal Environmental Research Laboratory develops new and improved technology and systems for the prevention, treatment, and management of wastewater and solid and hazardous waste pollutant discharges from municipal and community sources, for the preservation and treatment of public drinking water supplies, and to minimize the adverse economic, social, health, and aesthetic effects of pollution. This publication is one of the products of that research; a most vital communications link between the researcher and the user community.

This report describes fundamental studies on methanogenic and hydrogenogenic microorganisms. Results demonstrate the existence of a new physiological group of bacteria which play a central role in anaerobic digestion of domestic waste.

Francis T. Mayo
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ABSTRACT

Methanogenic bacteria were isolated from mesophilic anaerobic digesters. The isolates were able to utilize H_2 and CO_2, acetate, formate and methanol, but were not able to metabolize propionate and butyrate. It was shown the propionate and butyrate are not substrates for methanogenic bacteria but are converted to hydrogen, carbon dioxide and acetate by a hydrogenogenic microflora. The reactions leading to methane were quantitatively analyzed. It was shown that acetate, propionate and butyrate metabolism were inhibited by hydrogen. The formation of acetate and propionate were shown to be rate limiting in the digestion process, and that sludge digestion was not inhibited by hydrogen under conditions of excess substrate.

This report was submitted in fulfillment of Grant No. 17070-DJV by the University of Florida under the sponsorship of the U. S. Environmental Protection Agency. This report covers the period September 1, 1966 to October 15, 1979 and work was completed as of October 15, 1979.

CONTENTS

FIGURES

TABLES

ACKNOWLEDGEMENTS

I thank Dr. R. A. Mah for his useful help in carrying out these experiments and acknowledge the contributions of Dr. D. Boone, Mr. F. Bordeaux, Dr. P. Shuba and Mrs. J. Ward to the project.

SECTION 1

INTRODUCTION

In 1776 the Italian physicist, Alessandro Volta, observed a combustible gas formed from plant material in lake sediments. Since that time many investigators have endeavored to elucidate the biological features of the formation of such gas. The gas has been shown to be methane. Practical interest in the methanogenic process later centered primarily on the formation of methane in the rumen of herbivorous animals and the utilization of the process in anaerobic waste treatment. Recently, further interest in the fermentation has been stimulated by its possible application to production of methane as a fuel source.

Understanding of methanogenesis as a dissimilation process remained in a primative state compared to our understanding of other major microbiological processes, because of the fastidious growth characteristics of the bacteria involved and our misunderstanding of the substrates used by these organisms and the products they produce. In addition, the taxonomy of these organisms is complicated by the paucity of their identifying characteristics.

A generalized scheme for the formation of methane from insoluble organic matter is shown in Figure 1. Prior to this investigation there have been no definitive studies showing whether this scheme is or is not correct. The studies reported here show the scheme to be incorrect. The investigations to be reported deal primarily with the question of the microbiology of methane production shown in the last two boxes. If the scheme shown in Figure 1 were correct the following would be possible:

1. Volatile acids and hydrogen should exist as intermediates in the process and it should be possible to chemically demonstrate their existence.

2. It should be possible to isolate, in pure culture, bacteria capable of converting the postulated intermediates to methane.

3. It should be possible to quantitate the contribution of each volatile acid to total methane formation and it should not be possible to quantitatively identify intermediates in an external pool between volatile acids and methane gas.

This investigation deals with the above considerations in methane production by mesophilic digestion of domestic organic wastes. In addition the investigation deals with the identification of the steps in the reaction, and considers the matter of digestion failure under conditions of excess substrate.

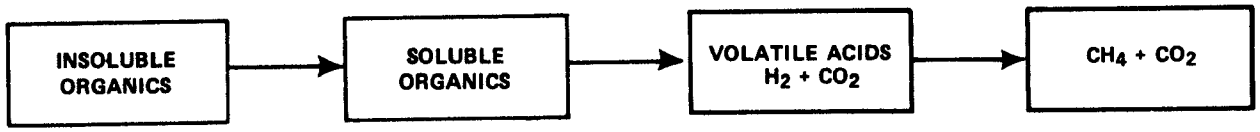

Figure 1. Conventional scheme for the formation of methane from insoluble organic matter based on previously known facts.

SECTION 2

CONCLUSIONS

1. The scheme shown in Figure 1 is incorrect. A correct scheme is shown in Figure 2.

2. A large and diverse methanogenic bacterial population exists in domestic sludge digesters. These organisms are restricted in their substrate utilization to the utilization of hydrogen, formate, methanol and acetate.

3. The results of this work demonstrate the existence of a new physiological group of bacteria which play a central role in anaerobic digestion of domestic waste. There exists hydrogenogenic bacteria which produce hydrogen from propionic acid and butyric acid. The dissimilation of these acids is not a methanogenic process. These hydrogenogenic organisms dissimulate as much as 70% of the organic matter in the eventual formation of methane.

4. Acetate and hydrogen plus carbon dioxide are the direct precursors of methane during anaerobic digestion.

5. Propionate and butyrate are the primary precursors of acetate and hydrogen during anaerobic digestion.

6. Hydrogen can inhibit the digestion process but is not the cause of digestion failure induced by excesses of organic substrates.

7. During normal digestion processes the rate of methane formation is limited by the rate of volatile acid production from other organic substrates.

8. Ethanol and iso-valerate are not intermediates in the digestion.

9. Iso-butyrate is quantitatively a minor intermediate.

10. The following contributions of volatile acids to methane as a final product in mesophilic digestion of domestic sludge were observed, calculated on maximum and minimum contributions of propionate and butyrate.

a.	Acetate*	-	53% : 24%
b.	Propionate	-	23% : 30%
c.	n-Butyrate	-	10% : 40%
d.	iso-Butyrate	-	1.5%

* Acetate not formed from propionate or n-butyrate.

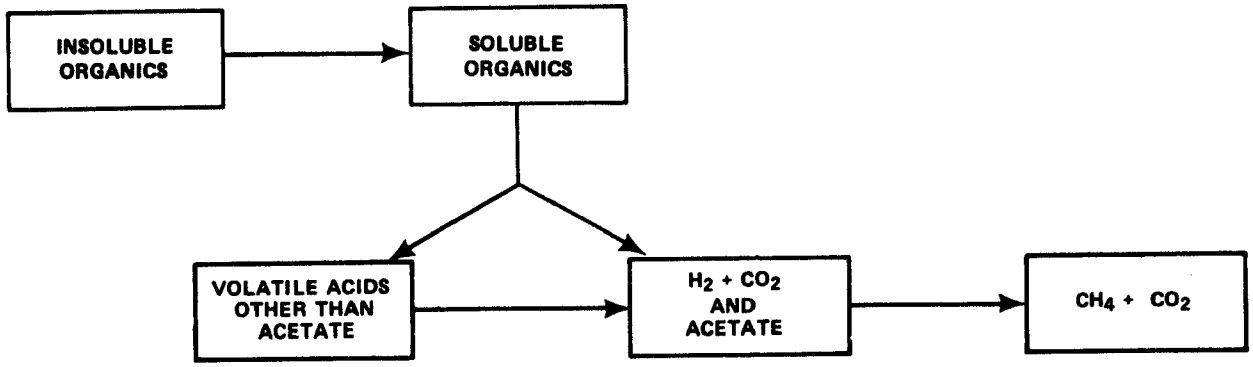

Figure 2. Scheme for the formation of methane from insoluble organic matter based on the data presented in this report.

SECTION 3

RECOMMENDATIONS

1. It is recommended that research be initiated to determine the biological characteristics of the hydrogenogenic microflora which function in anaerobic digestion with emphasis on those factors which stimulate growth and survival of these unique organisms.

SECTION 4

RESULTS AND DISCUSSION

ISOLATION OF PURE CULTURES

Materials and Methods used are presented in Appendix A. During the course of this investigation a major effort was made to isolate, in pure culture, those bacteria which were responsible for methane production in anaerobic digestors. At the beginning of these studies it was assumed that the immediate precursors for methanogenesis were short chain volatile acids, hydrogen, and carbon dioxide. It was assumed that bacteria such as Methanobacterium propionicum and Methanobacterium suboxydans did in fact exist, and their isolation in pure culture awaited only the application of proper techniques. Hydrogen utilizing methanogens and an acetate utilizing methanogen were readily isolated. All efforts to isolate propionate and butyrate dissimilating methanogens were unsuccessful. Each effort in this regard ended with the isolation of a hydrogen oxidizing methanogen, in most cases an organism now know as Methanospirillum hungatii. The failure of this massive attempt led to an experimental study of the possibility that short chain volatile organic acids were indirectly metabolized by a hydrogenogenic and a methanogenic microflora. If this were the case, then organisms such as Methanobacterium propionicum and Methanobacterium suboxydans would not exist. Data presented later in the report demonstrates that this is the case. Propionate and butyrate are metabolized by a hydrogenogenic microflora.

The isolation and characterization of methanogens has been unsatisfactory until very recently because of the fact that substantial distinguishing features could not be obtained for these organisms. The biology of this question has been recently reviewed by Wolfe and Higgins (27). The basis for the confusion associated with the taxonomy of these organisms has recently been identified by Balch et al. (1). Application of the 16 S r RNA analysis methods developed by Woese have shown that these organisms are only distantly related to typical bacteria. They are different from other forms of microorganisms studied in the past.

An effort was made to obtain conventional characters for classification of these organisms. Initial experiments were positive, but later results failed to confirm the initial observations. Isolation results are shown in Table 1. Isolate 2 is now known in the literature as Methanobacterium ruminantium strain P.S. This is the most prevalent methanogen in digesting sludge.

TABLE 1. CHARACTERISTICS OF ISOLATES

Characteristics	Isolate 1	2	3	4	5	6
Morphology	Rod	Rod	Sarcina	Rod	Coccus	Rod
Length	Variable	1.8 μ	4 μ	5 μ	1.5 μ	2.0 μ
Width	0.4 μ	0.7 μ	4 μ	1.0 μ	1.5 μ	0.5 μ
Motility	-	-	-	-	-	-
Capsule	-	+	-	-	-	-
Spore formation	-	-	-	-	-	-
Anaerobic growth	+	+	+	+	+	+
Aerobic growth	-	-	-	-	-	-
Catalase production	-	-	-	-	-	-
Nitrate reduction	-	-	-	-	-	-
Sulfate reduction	-	-	-	-	-	-
CO_2 reduction	+	+	+	+	+	+
Gram reaction	+	+	+	+	+	+
Growth on hydrogen	+	+	+	+	+	+
Growth on formate	+	+	-	+	+	+
Growth on acetate	-	-	+	-	-	-
Growth on methanol	-	-	+	-	-	-
Growth on ethanol	-	-	-	-	-	-
Growth on sugars	-	-	-	-	-	-
Growth on mineral medium	+	-	±*	-	-	+
Growth at 55°C	-	-	-	-	-	-
Growth at 45°C	+	+	+	+	+	+
Growth at 30°C	+	+	+	+	+	+
Growth at 25°C	-	-	-	-	-	-
CH_4 from CO_2 and H_2	+	+	+	+	+	+
CH_4 from formate	+	+	-	+	+	+
CH_4 from acetate	-	-	+	-	-	-
CH_4 from methanol	-	-	+	-	-	-
CH_4 from sugar	-	-	-	-	-	-
CH_4 from glycine	-	-	-	-	-	-
CH_4 from alanine	-	-	-	-	-	-
CH_4 from serine	-	-	-	-	-	-
CH_4 from glycerol	-	-	-	-	-	-
CH_4 from tartrate	-	-	-	-	-	-
CH_4 from isovalerate	-	-	-	-	-	-
CH_4 from raw sludge	-	-	-	-	-	-

± *, Varies with different strains.

Isolate 4 is now known as Methanspirillum hungatii (7).
Isolates 1 and 6 were cultures of Methanobacterium formicicum.
Isolate 3 is a strain of Methanosarcina barkeri.
Isolate 5 has been lost. Efforts will be made to re-isolate.

In addition to the above isolates, a salt tolerant coccus was isolated which was believed to be Methanococcus vannielii. It has, however, turned out to be a species distinct from M. vannielii.

These organisms, along with organisms isolated by other investigators, are to be characterized shortly in a new taxonomic scheme based on the 16S r RNA concept (Personal communication R.S. Wolfe). This taxonomy will, for the first time, place the methanogenic bacteria in a rational scheme. Three of the fourteen organisms to be characterized will be organisms isolated during the course of this study. They are isolate 2 and 4 and the salt tolerant coccus. Isolates 2 and 4 were obtained from digesting sludge and are present there in numbers exceeding 1×10^7/ml. The coccus was isolated from an estuary and is not believed to be an inhabitant of digesting sludge.

The results show that hydrogen utilizing methanogens are present in large numbers in digesting sludge. As shown later in this report, they function to maintain a low hydrogen concentration in digesting sludge.

DETERMINATION OF METHANOGENIC INTERMEDIATES

Kluyver (11) elucidated the biological principle that the metabolism of living organisms involves a continuous and directed flow of electrons from electron doners to electron acceptors. Metabolic processes terminate when electron transfers cease. Considered from this point of view sludge digestion consists of a continuous and orderly flow of electrons which produces methane, carbon dioxide, and cells, since these are the terminal products of electron transfer under the operating conditions of a sludge digestor. These same products are produced during the dissimilation of organic matter in other anaerobic environments. The reactions in a sludge digestor occur in a habitat fairly constant in temperature, pH, anaerobiosis, and substrate availability. The dissimilation rate is rapid, the pathways varied, and the microflora diverse but fairly constant. These characteristics make sludge digestors attractive as a source material for ecological studies, with the prospect that information obtained from this source will be applicable to the understanding of other important environments such as swamps and lake sediments.

Sludge digestion has been generally believed to consist of three steps: hydrolysis of complex materials, acid production, and methane formation. The microbiology and biochemistry of these steps have not been clearly elucidated. Jeris and McCarty (10) have shown that methane evolved from enrichments could be accounted for primarily on the basis of the dissimilation of acetic and propionic acid.

The rapid reaction rates of sludge methanogenesis make sludge an attractive material to analyze directly using isotope dilution techniques. The

possible role of acetate in sludge methanogenesis was investigated, using these techniques, by Smith and Mah (22). Some of the data from these studies are included for clarity, because they were conducted under a previous portion of this grant.

A brief discussion of theoretical considerations is included here because erroneous interpretations have been recently published. Strayer and Tiedje (21) concluded that, using this technique, the H_2 contribution to methane projection could be lost in the gas headspace. They failed to recognize that this event would cause the digestion to deviate from a steady state and since steady state conditions were maintained, H_2 loss could not have occurred. Analysis of the gas phase from these systems had in fact been analyzed, but not reported because the question had been eliminated with the control showing a steady state. The H_2 concentration in the digester vessels varied from 0.003% to 0.009%, for gases containing 60% to 66% CH_4.

The rate of dissimilation of an intermediate in methane formation is the product of pool size times the turnover rate. The turnover rate can be determined, under certain circumstances, from the rate of dilution of added radioactive tracer. This is governed by the following consideration.

$$\frac{dx}{dt} = -kx$$

t = time
x = total radioactivity in the intermediate
k = the rate constant

then:

$$\int_{x_o}^{x_t} \frac{dx}{x} = -k \int_{t_o}^{t} dt$$

and: $\ln x_t = -kt + \ln x_o$

This equation is simply the equation for a straight line with a slope of m.

$$y = mx + b.$$

The rate constant k equals minus m.

This then means that for experiments to be valid, the reactions must occur with gas evolution being constant, the pool size of the intermediates constant, and the plot of ln radioactivity (in some form proportional to specific activity) against time linear. Controls must be included in the experimentation to show that such is the case. If dilution of the intermediate occurs by some mechanism other than the formation of non-radioactive molecules from non-radioactive precursors, the experiments are not valid. Deviation may occur from an exchange reaction, from a change in pool size due to the addition of the intermediate, or from alterations in the steady state of the reaction sequences. In the work reported here steady states were maintained, except as noted.

Formate

It was postuated that formate could play an important role in sludge methanogenesis. An effort was made to evaluate this possibility using isotope dilution techniques. Results are shown in Figure 3. Linear dilution of formate was not observed. The formate exchanged rapidly with CO_2. The method is therefore not applicable. Efforts to redesign the experimental procedure failed because the rate of the exchange reaction was the same as the rate of formate dilution, within the limits of our experimental procedures. The possible role of formate in sludge methanogenesis is not known.

Acetate

Acetate turnover was calculated using the procedures described in the methods section. Figure 4 shows that the rate of gas evolution during the experiments was constant. Figure 5 shows that our batch fed digesters had constant pool sizes of acetic acid during a period of from 3 to 7 hours after feeding. Figure 6 shows that the rate of isotope dilution was linear. Manometric measurements, and direct analysis of the gas phase showed that gas evolution was constant during the course of the experiment. Similar results were observed for the other experiments.

The rate of methane production during the experiment was 0.033 μmoles/ml/min. The rate constant from Figure 6 is 0.0052/min and the acetic acid pool was 4.7 μmoles/ml. Assuming 1 mole of methane from 1 mole of acetate, the acetic acid would account for approximately 73% of the methane produced. The results of a similar experiment, with similar controls, are shown in Figure 7. In this experiment the rate of methane production was 0.042 μmoles/ml/min. The rate constant from Figure 7 is 0.0078/min and the acetic acid tool was 3.8 μmoles/ml. Again calculating from methane rate and pool size times the rate constant, approximately 71% of the methane produced would pass through acetate.

Propionate

Propionate turnover was calculated using the same procedures as used for acetate. Figure 8 shows the results of such an experiment. The pool size during the experiment was 0.87 μmoles/ml, the rate constant from the graph is 0.0041/min and the rate of methane formation during the experiment was 0.021 μmole/ml/min. The rate of turnover of propionate calculated from this is 0.0036 μmoles/ml/min. Assuming 1.75 moles of methane per mole of propionate, metabolized propionic acid would contribute 30% of the total methane formed, 13% via CO_2 reduction and 17% via acetate.

Figure 9 shows the results of a second experiment. In this experiment the pool size was 1.4 μmoles/ml, the rate constant 0.0039/min and the rate of methane formation during the experiment 0.042 μmoles/ml/min. The rate of propionate turnover calculated from this is 0.0055 μmoles/ml/min. Assuming 1.75 μmoles of methane from one mole of propionate, propionate would contribute 23% of the total methane formed, 10% via CO_2 reduction and 13% via acetate. Another experiment gave results showing 27% of the total

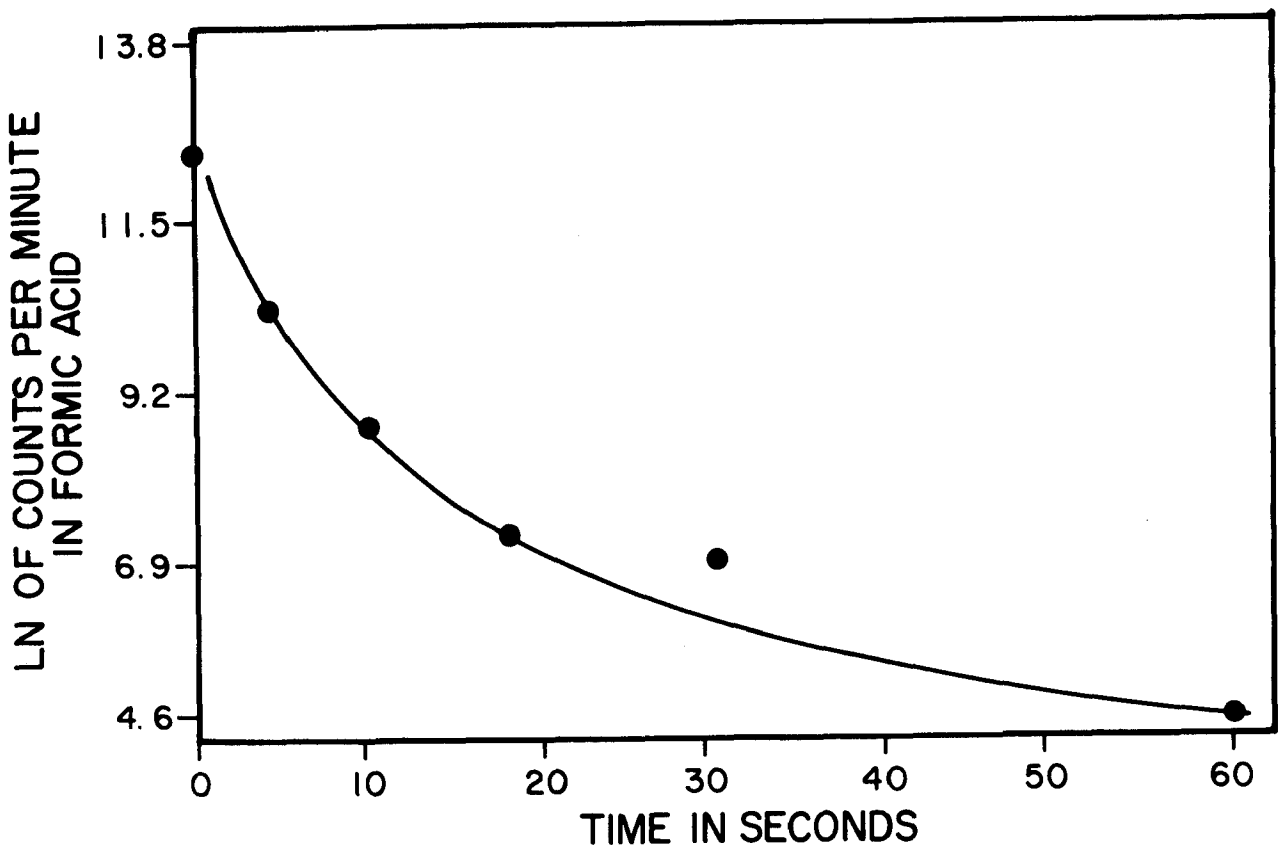

Figure 3. Change in counts per minute in the formic acid fraction from a constant volume of sludge, after addition of radioactive formic acid.

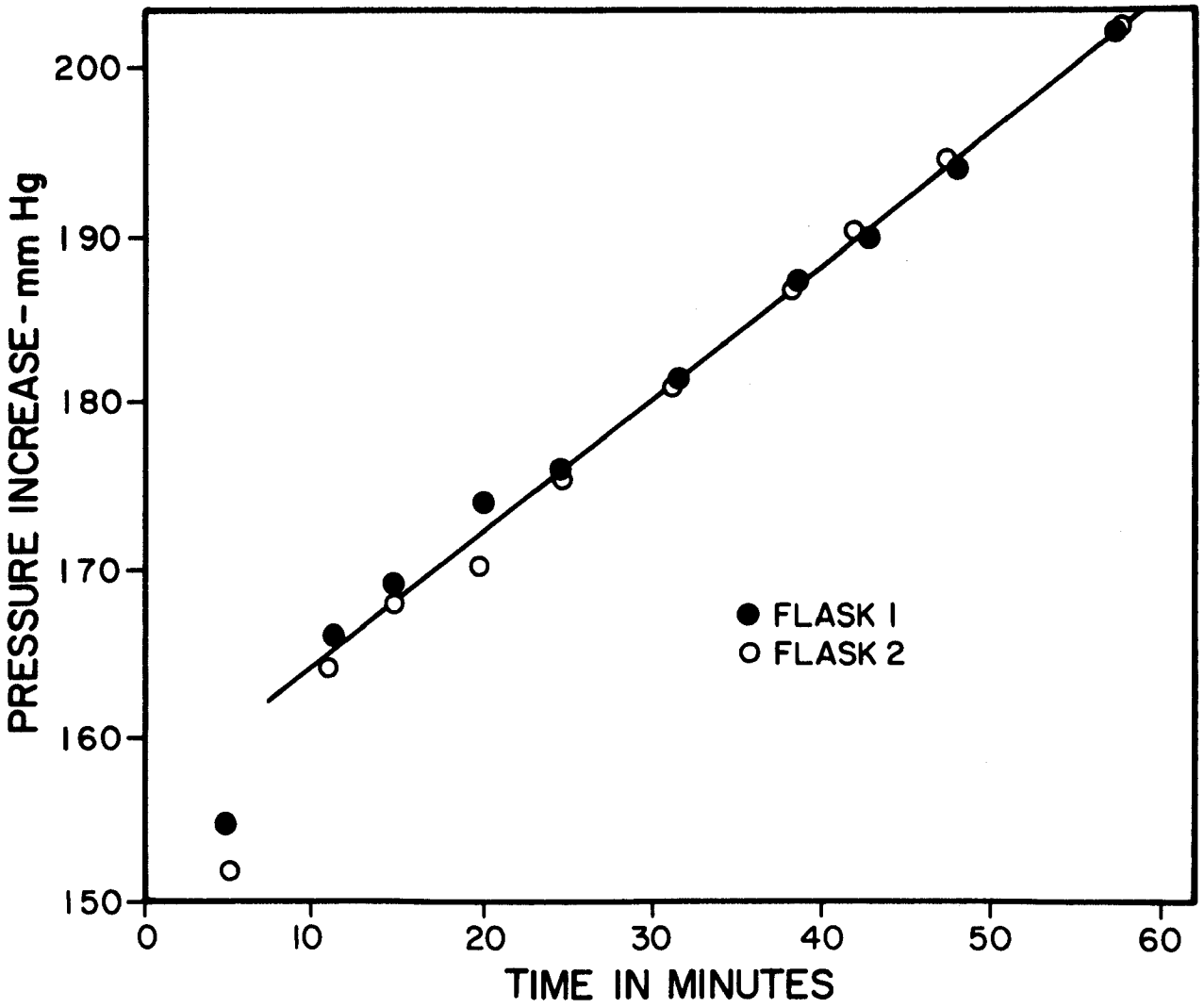

Figure 4. Manometric changes in duplicate control flasks during the period of the experiment. These flasks contained no add isotopes.

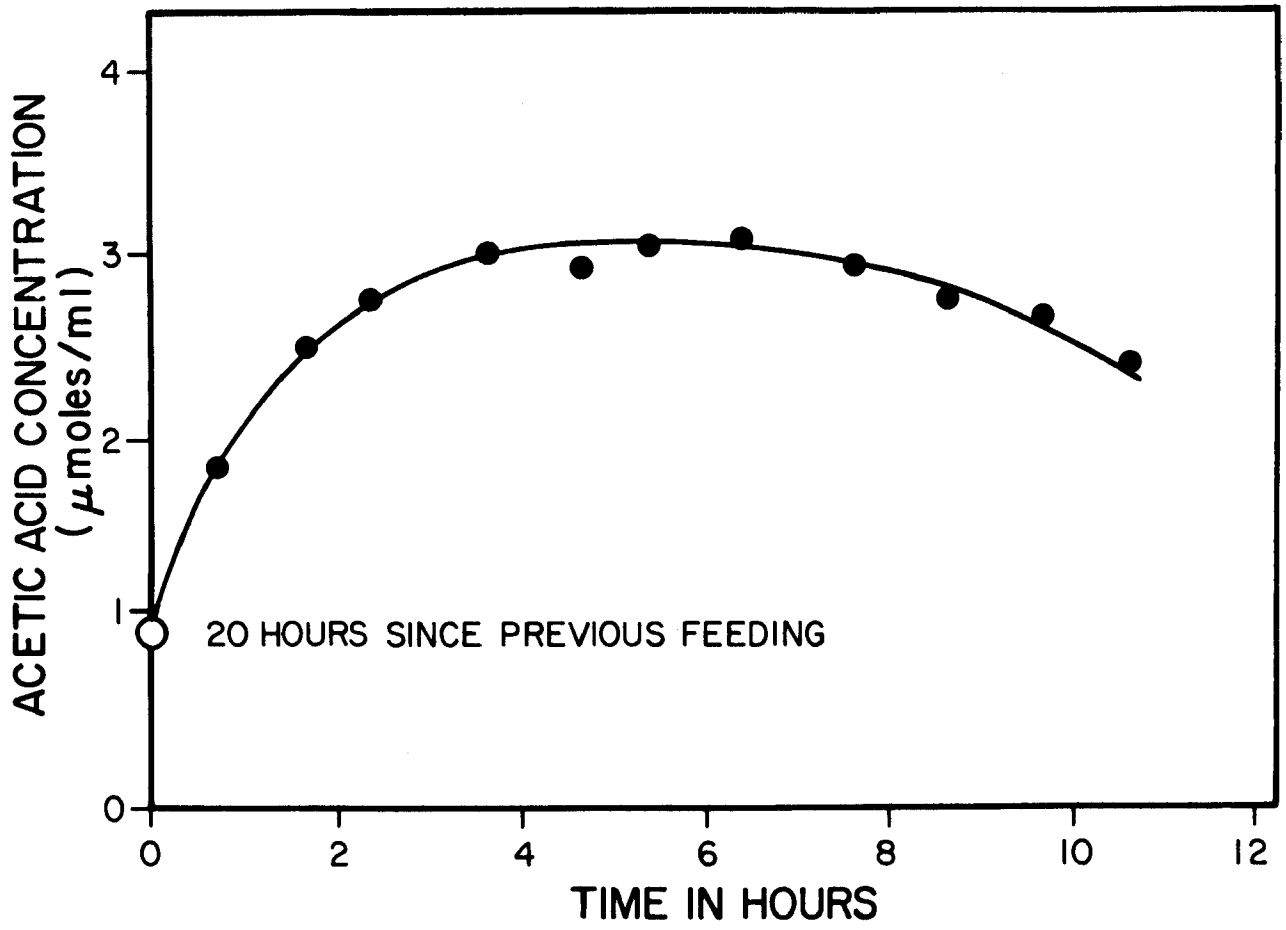

Figure 5. Changes in the acetic acid concentration of sludge during an eleven hour period following the feeding of the digester.

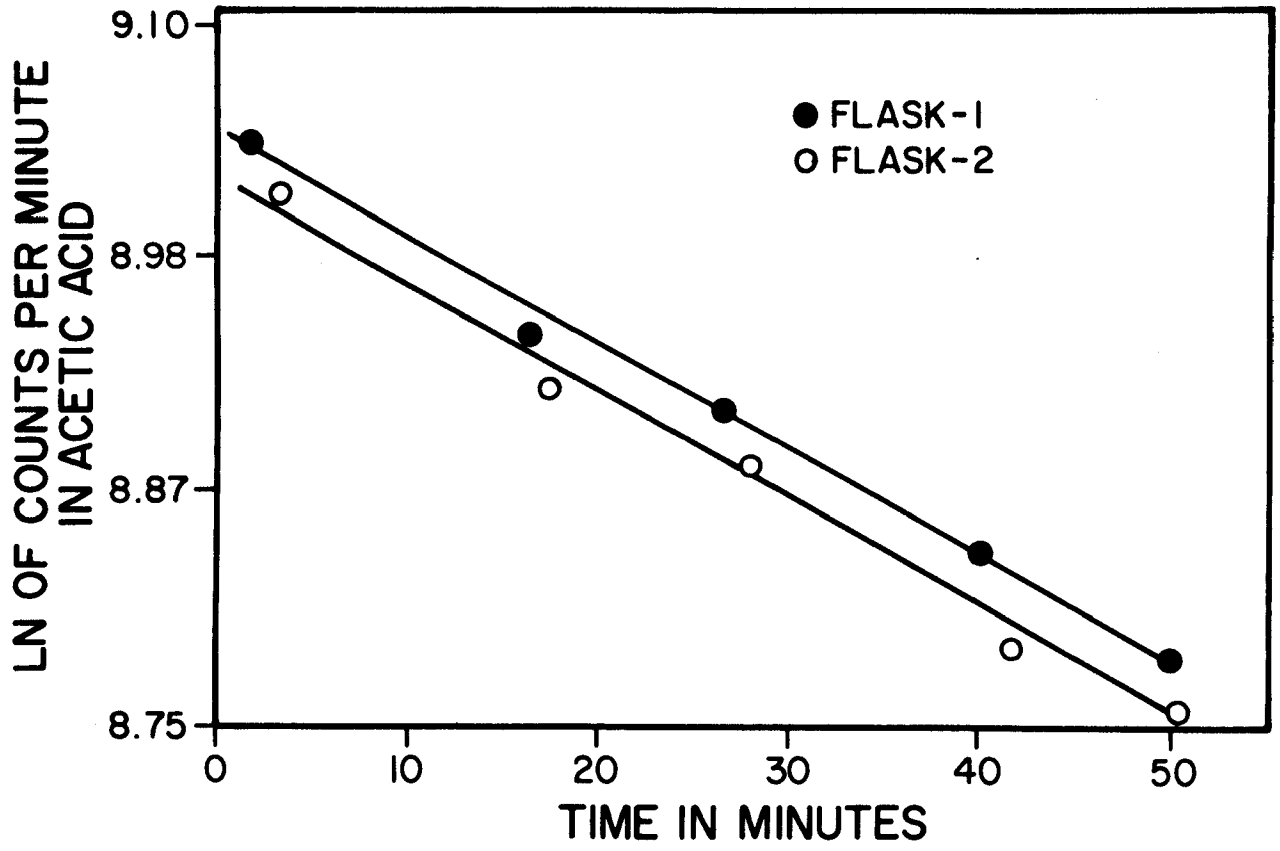

Figure 6. Change in counts per minute in the acetic acid fraction from a constant volume of sludge, after addition of radioactive acetic acid to duplicate flasks containing sludge.

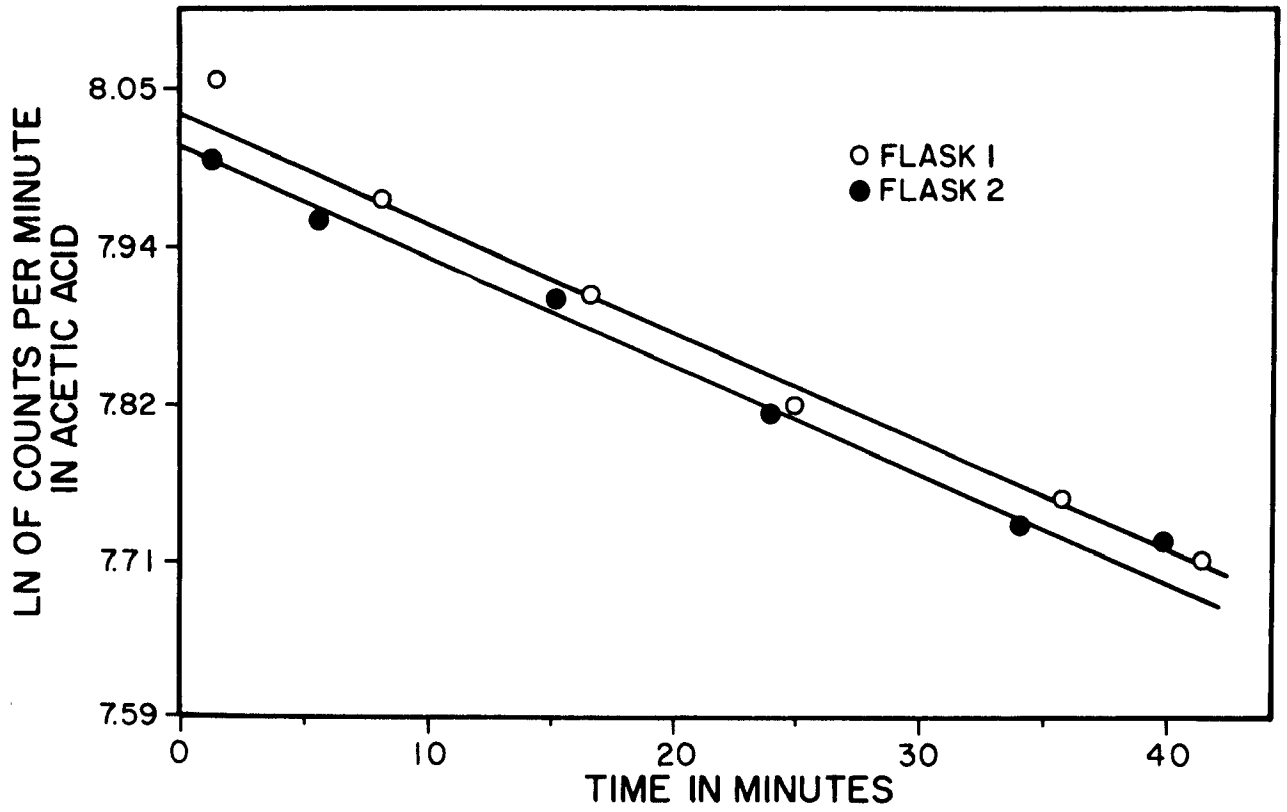

Figure 7. Change in counts per minute in the acetic acid fraction from a constant volume of sludge, after addition of radioactive acetic acid to duplicate flasks containing sludge.

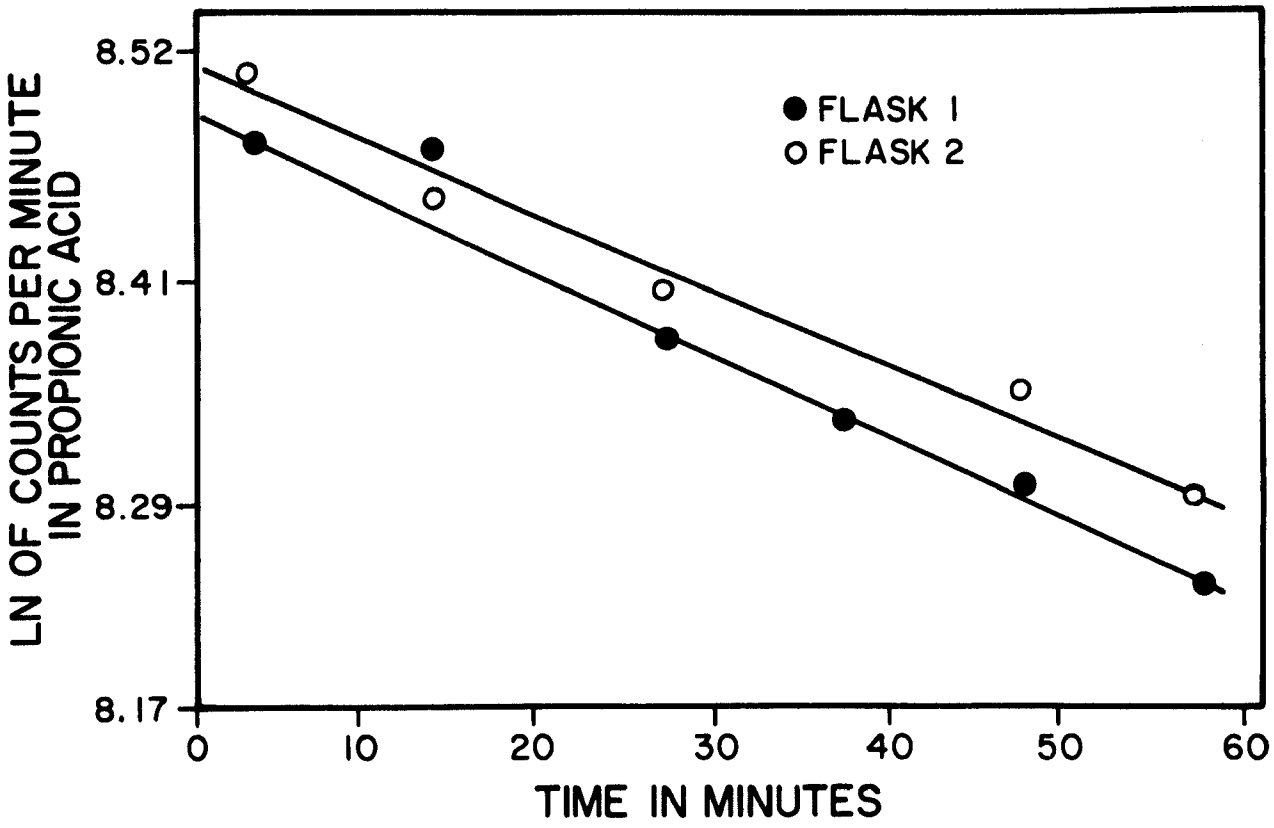

Figure 8. Change in the counts per minute in the propionic acid fraction from a constant volume of sludge, after addition of radioactive propionic acid to duplicate flasks containing sludge.

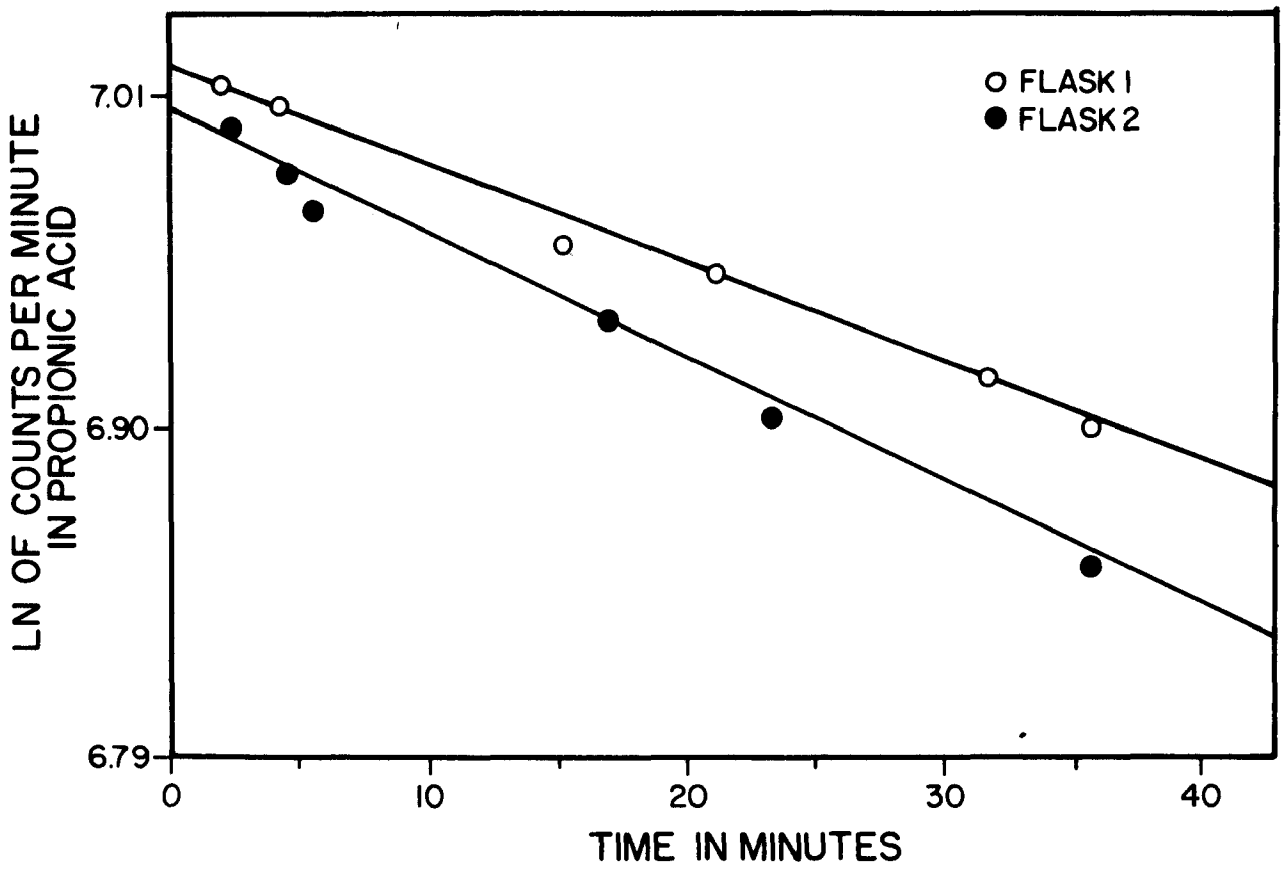

Figure 9. Change in the counts per minute in the propionic acid fraction from a constant volume of sludge, after addition of radioactive propionic acid to duplicate flasks containing sludge.

methane formed through propionate, presumably 16% via acetate and 11% via CO_2 reduction.

n-Butyrate

Experiments were conducted with n-butyric acid, utilizing the methods previously described, with results shown in Figure 10. In this experiment the pool size of n-butyrate was 0.14 μmoles/ml, the rate constant 0.033/min, and the rate of methanogenesis 0.029 μmoles/ml/min. The butyrate turnover would then be 0.0046 μmoles/ml/min. Assuming 2.5 moles of methane per mole of n-butyrate, n-butyrate would contribute 40% of the total methane formed, 8% by CO_2 reduction and 32% by way of acetate.

A similar experiment was conducted with sludge from a digester producing methane at a rate of 0.0043 μmoles/ml/min, with results shown in Figure 11. The pool size was 0.067 μmoles/ml, and the rate constant 0.026/min. These results give a turnover of butyrate of 0.0017 μmoles/ml/min, which calculate to a 10% contribution of butyrate to methane, approximately 3.3% by CO_2 reduction and 6.6% by way of acetate.

iso-Butyrate

Low, but consistant, pools of iso-butyrate were observed. An experiment was conducted, with considerable difficulty as shown by the spread of points on Figure 12. The sludge used produced methane at a rate of 0.047 μmoles/ml/min. The pool size was 0.021 μmoles/ml. The rate constant calculated from Figure 12 was 0.013/min giving a turnover rate for iso-butyrate of 0.00027. Assuming 2.5 μmoles of methane per mole of iso-butyrate, iso-butyrate would contribute 1.5% of the total methane formed.

Ethanol

Ethanol added to sludge disappeared very rapidly as shown in Figure 13. However, when radioactive ethanol was added, no appreciable change in specific activity was observed over a 35 minute period as shown in Table 2. Therefore, ethanol is not produced in this fermentation and does not contribute to the total methane formed.

TABLE 2. TURNOVER OF ETHANOL IN SLUDGE

Time (min.)	Specific Activity picocuries/μmole
1	6.2
10	6.7
20	7.0
35	6.6

The variation observed was assumed to be due to analytical error.

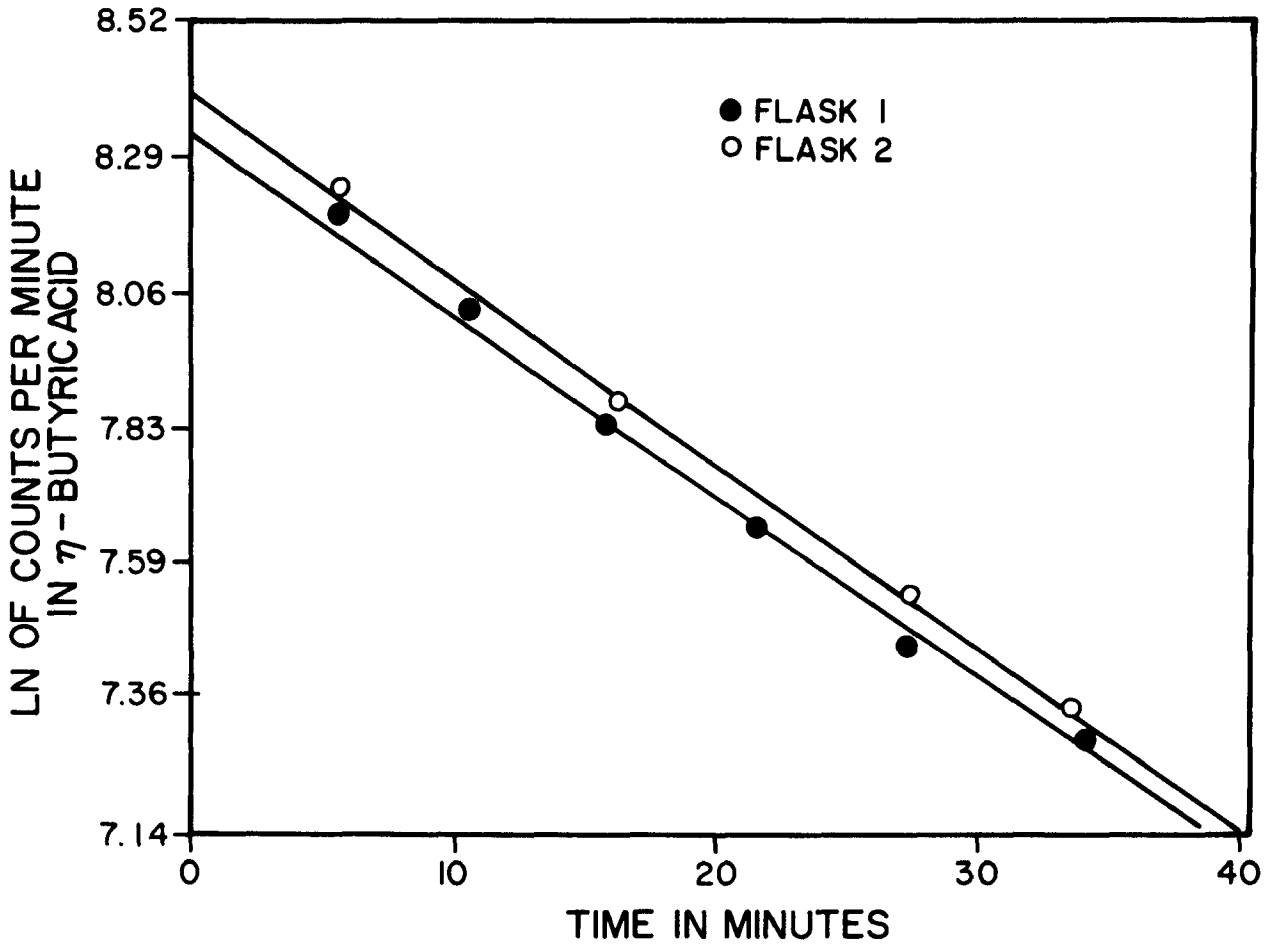

Figure 10. Change in the counts per minute in the n-butyric acid fraction from a constant volume of sludge, after addition of radioactive n-butyric acid to duplicate flasks containing sludge.

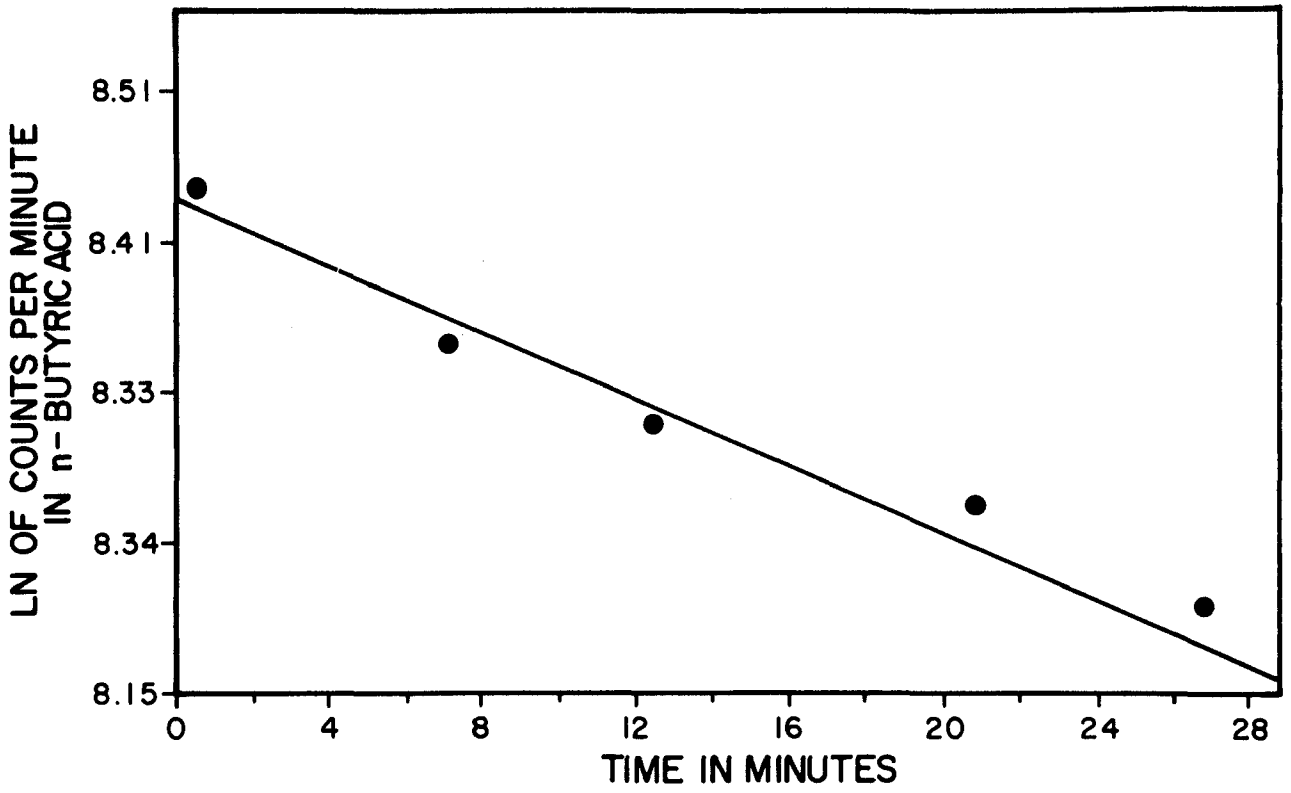

Figure 11. Change in the counts per minute in the n-butyric acid fraction from a constant volume of sludge, after addition of radioactive n-butyric acid.

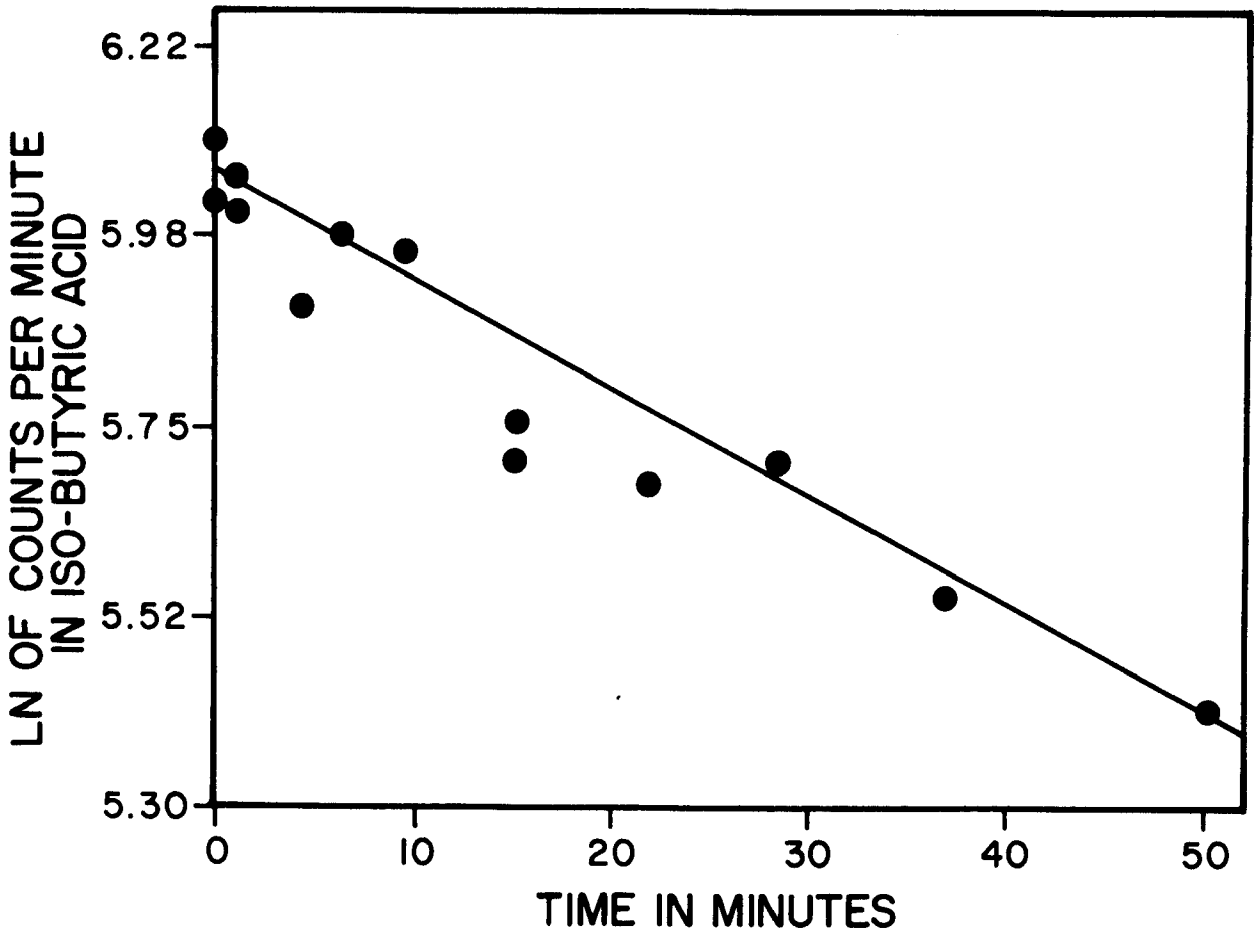

Figure 12. Change in the counts per minute in the iso-butyric acid fraction from a constant volume of sludge, after addition of radioactive iso-butyric acid.

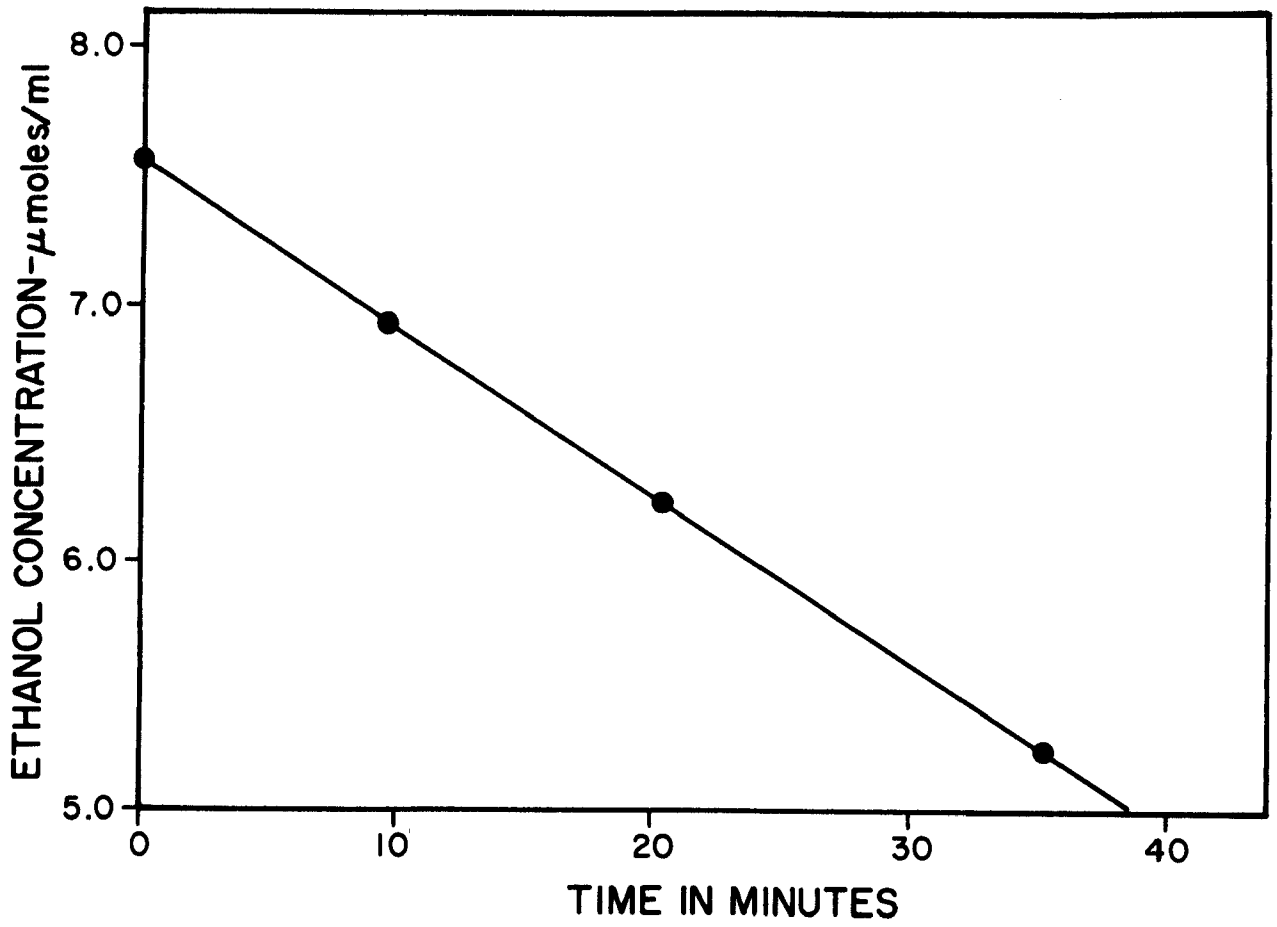

Figure 13. Change in the concentration of ethanol added to sludge, after addition of ethanol.

iso-Valeric Acid

Iso-valeric acid was observed in low concentrations in the digesting sludge. However, as with ethanol, there was no change in specific activity upon addition of label as shown in Table 3. This demonstrates that iso-valeric acid does not contribute appreciably to the production of methane.

TABLE 3. TURNOVER OF ISO-VALERIC ACID IN SLUDGE

Time (min.)	Counts/min/ml in iso-valerate
0	4.9×10^5
5	5.0×10^5
75	4.8×10^5

CONVERSION OF PROPIONATE AND BUTYRATE TO ACETATE

Experiments were conducted using techniques similar to those used for the turnover experiments to evaluate the validity of the concept of conversion of propionate and butyrate to acetate. ^{14}C-labeled propionate was added. Samples were removed and the amount of label in acetate and propionate per unit of volume was determined with the results shown in Figure 14. A similar experiment was conducted with ^{14}C-butyrate with the results shown in Figure 15. The expected values were calculated from the rates of the previous turnover experiments. The results are consistent with the conversion of propionate and butyrate to acetate.

HYDROGEN EFFECTS ON SLUDGE DIGESTION

Inhibition of Volatile Acid Turnover

The large number of methanogenic bacteria in digesting sludge suggested that the capacity of digesting sludge for hydrogen metabolism should be great. The fact that all isolates utilized hydrogen suggested that addition of hydrogen to digesting sludge should stimulate the methanogenic process since addition of hydrogen should result in an increase in the population of the methanogenic bacteria. Capacity of digesting sludge for hydrogen metabolism was determined by incubating digesting sludge under a gas phase of 70% hydrogen and 30% carbon dioxide. Hydrogen uptake and methane formation were determined quantitatively. Methane formation was also quantitatively determined for digesting sludge incubated under a gas phase of 70% nitrogen and 30% carbon dioxide. The results are shown in Table 4. Taking average values, 447 μmoles of hydrogen were utilized in the hydrogen flasks. Assuming 1 μmole of methane from 4 μmoles of hydrogen, 112 μmoles of methane would have been produced from added hydrogen and carbon dioxide, leaving 98 μmoles of methane formed from the sludge. However 134 μmoles of methane were produced in the nitrogen flasks. The difference between the 134 μmoles of methane produced from sludge in the absence of added hydrogen and

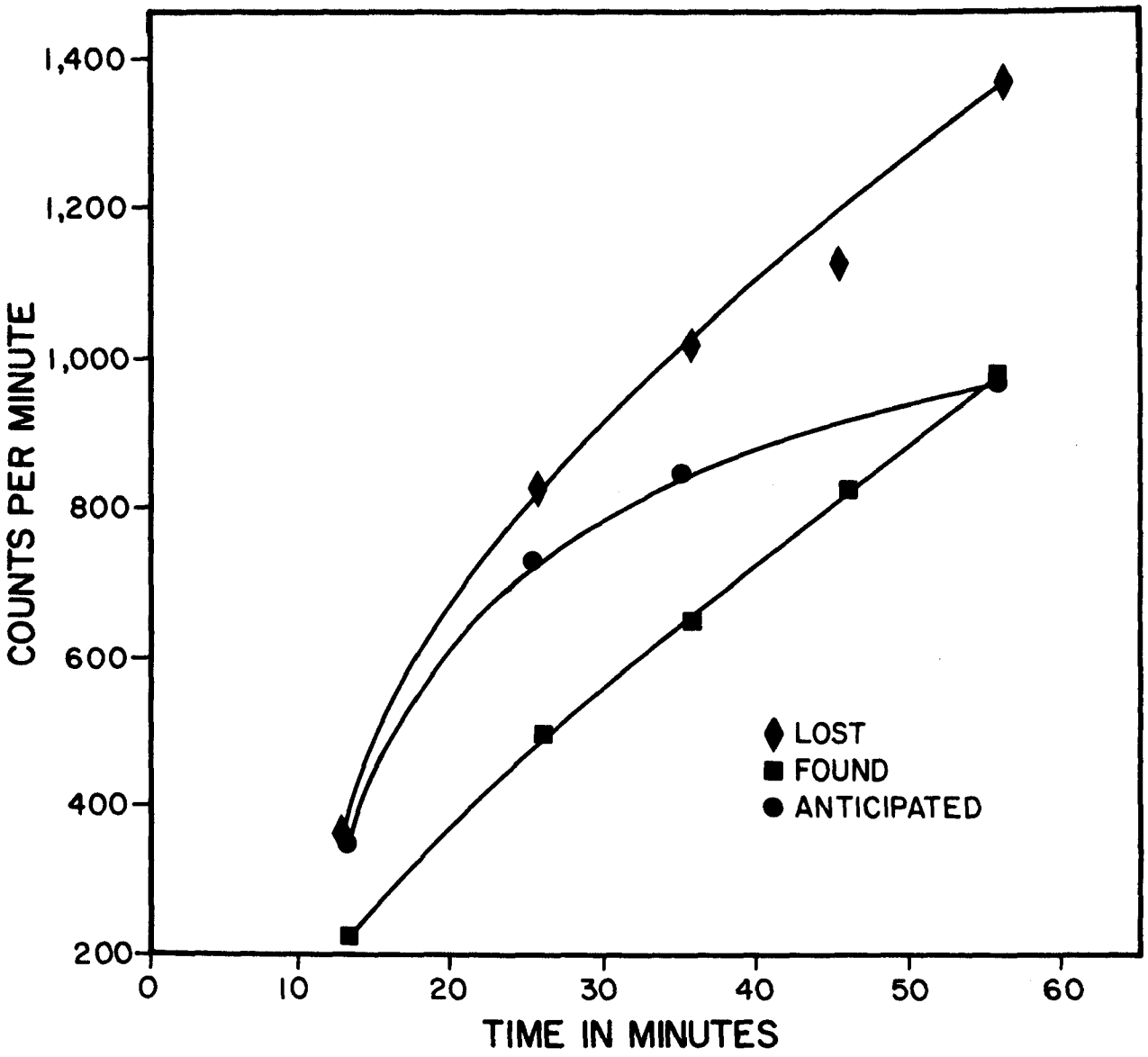

Figure 14. Counts per minute lost from propionic acid, and found in acetic acid, in a constant volume of sludge, following addition of radioactive propionic acid.

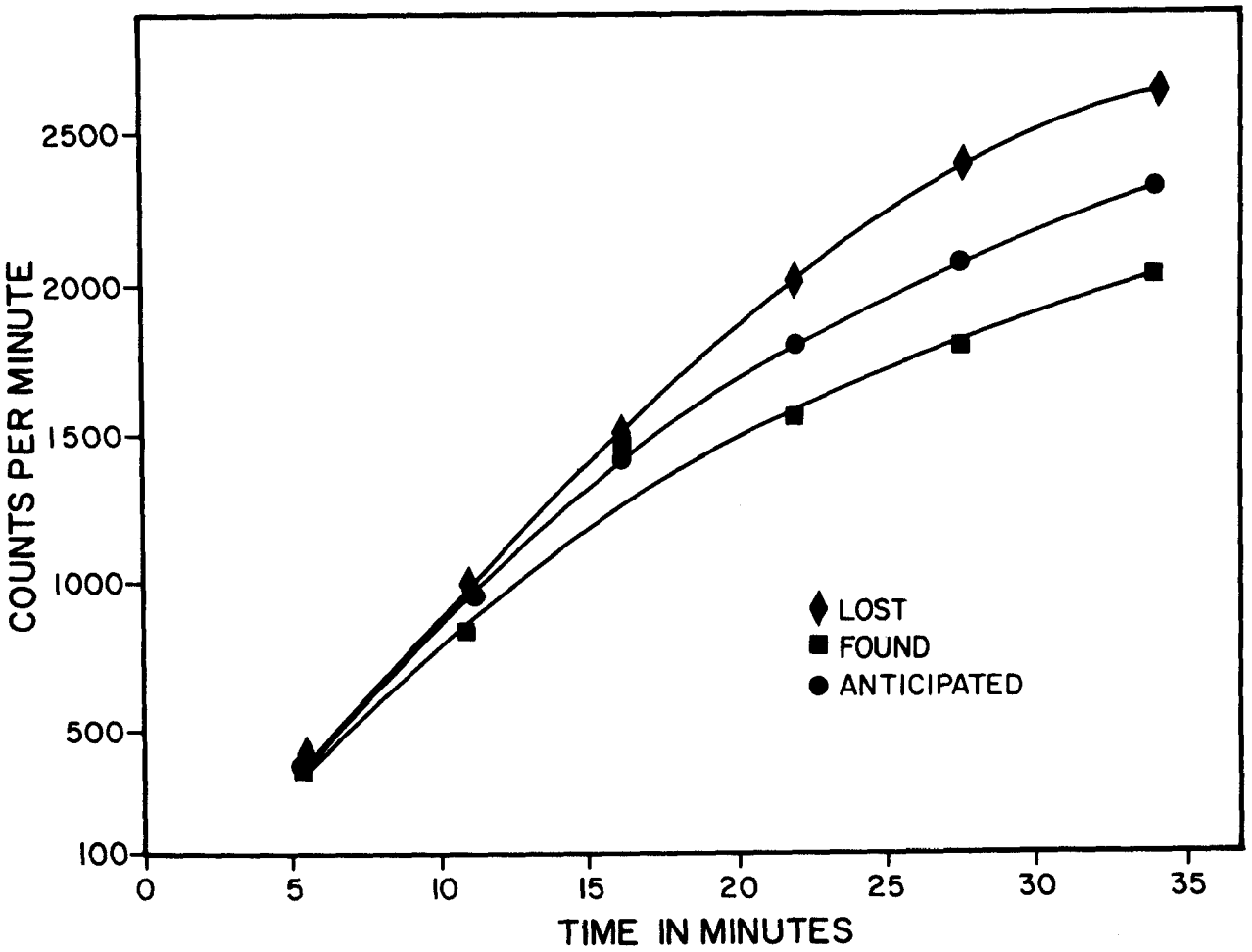

Figure 15. Counts per minute lost from butyric acid, and found in acetic acid, in a constant volume of sludge, following addition of radioactive butyric acid.

the 98 μmoles calculated from sludge in the presence of hydrogen indicates that either hydrogen inhibited methane production from other sludge precursors, or hydrogen and carbon dioxide were converted to molecules other than methane. The rate of methane formation was determined for sludge samples which had been equilibrated for two hours with a gas phase of 70% hydrogen and 30% carbon dioxide. This rate was compared to the rate of methanogenesis from sludge which had been equilibrated for two hours with a gas phase of 70% nitrogen and 30% carbon dioxide. The rates were calculated from the methane evolved during seventy-five minutes immediately following the equilibration period. The results are shown in Table 5. The lowered rate of methane evolution following exposure to hydrogen gas shows that an inhibition of methanogenesis had occurred. Calculations with average values from Table 5 indicate a 15% inhibition following exposure to a 70% hydrogen atmosphere. Calculations from average values of gas utilization and production given in Table 4 show an inhibition of 30% during exposure to a 70% hydrogen atmosphere. The difference between these two figures can be explained on the basis of recovery from inhibition following the brief exposure to molecular hydrogen during the experiment reported in Table 5.

The initial data obtained suggested that hydrogen gas should stimulate anaerobic digestion of sludge. However, initial experiments did not produce the anticipated result. In fact, hydrogen had an inhibitory effect. The inhibition was approximately 30%, consistent with the hypothesis that the inhibition was an inhibition of propionate, without an inhibition of acetate. To test this hypothesis, experiments were conducted to determine the effect of hydrogen on the rate constants for propionate and butyrate turnover with the results shown in Figure 16 and Figure 17. Hydrogen inhibited propionate turnover strongly but had little if any effect on acetate turnover. These experiments were conducted under a gas phase of 70% hydrogen, 30% carbon dioxide.

An experiment was then conducted to determine the concentration of hydrogen which inhibited the reaction. In this experiment the inhibition was followed by measuring the appearance of labeled acetate from labeled propionate in sludge incubated in equilibration with various concentrations of hydrogen. The gas mixtures used were 30% carbon dioxide, with a balance of hydrogen plus nitrogen. The results are shown in Figure 18. The fermentation was inhibited by 18% hydrogen but not 9% hydrogen.

TABLE 4. HYDROGEN UTILIZATION BY DOMESTIC SLUDGE

Gas Phase	CH_4 Formed in μmole	Initial H_2 in μmole	Final H_2 in μmole
70% H_2, 30% CO_2	207	1650	1220
" "	214	1695	1230
70% N_2, 30% CO_2	132	0	3
" "	136	0	3

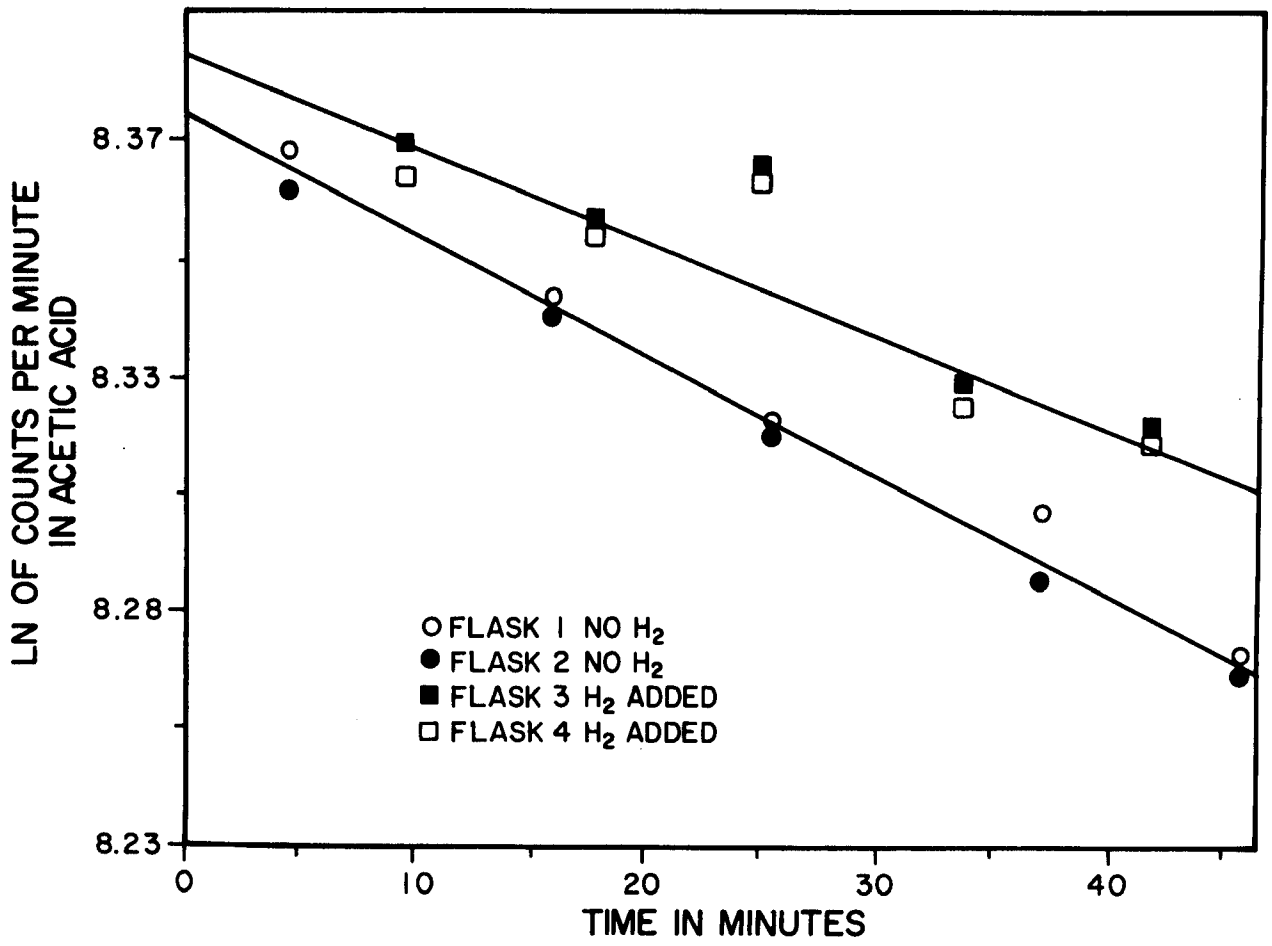

Figure 16. Change in counts per minute in the acetic acid fraction from a constant volume of sludge after addition of radioactive acetic acid to duplicate flasks containing sludge exposed to hydrogen, and duplicate flasks containing sludge not exposed to hydrogen.

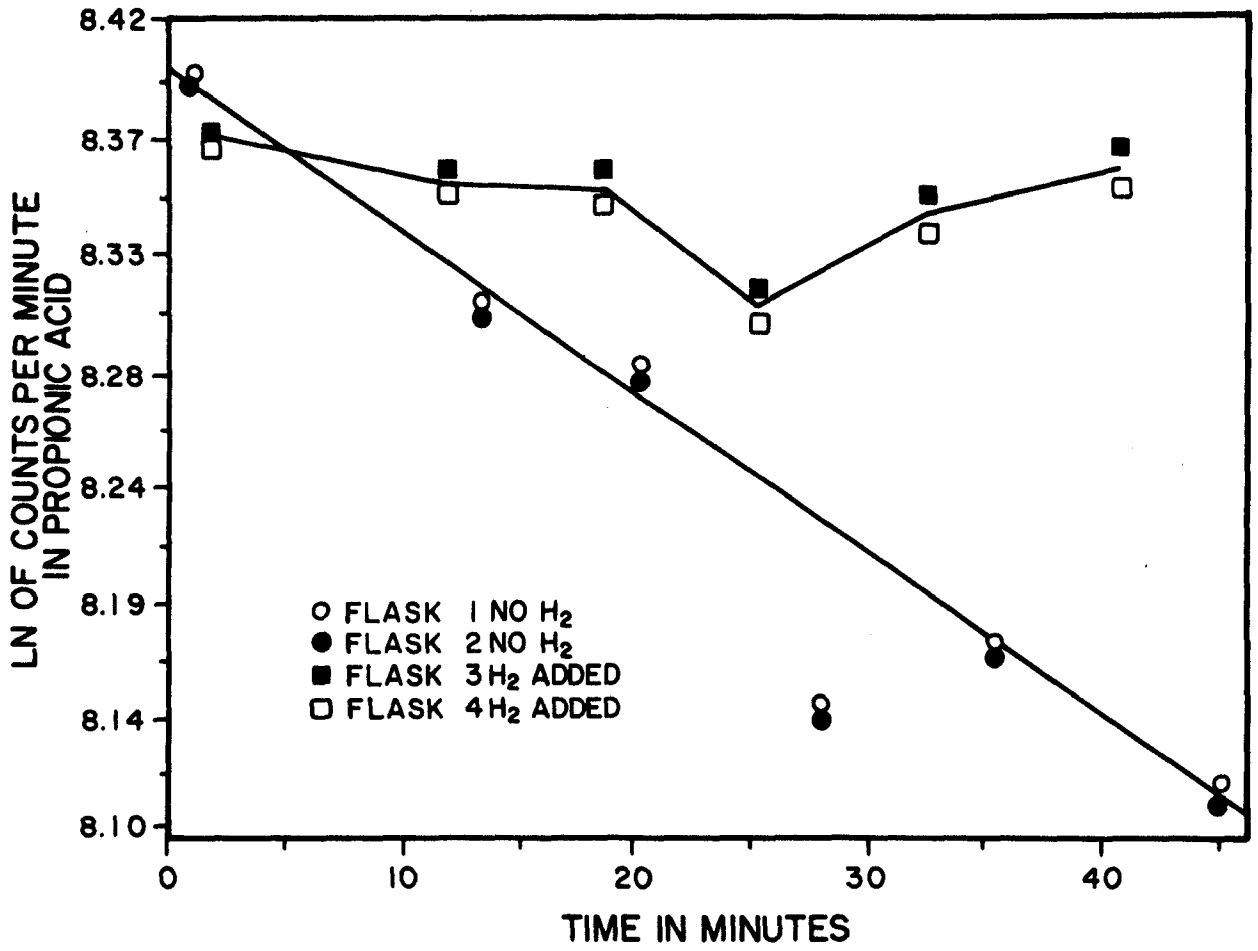

Figure 17. Change in counts per minute in the propionic fraction from a constant volume of sludge after addition of radioactive propionic acid to duplicate flasks containing sludge exposed to hydrogen, and duplicate flasks containing sludge not exposed to hydrogen.

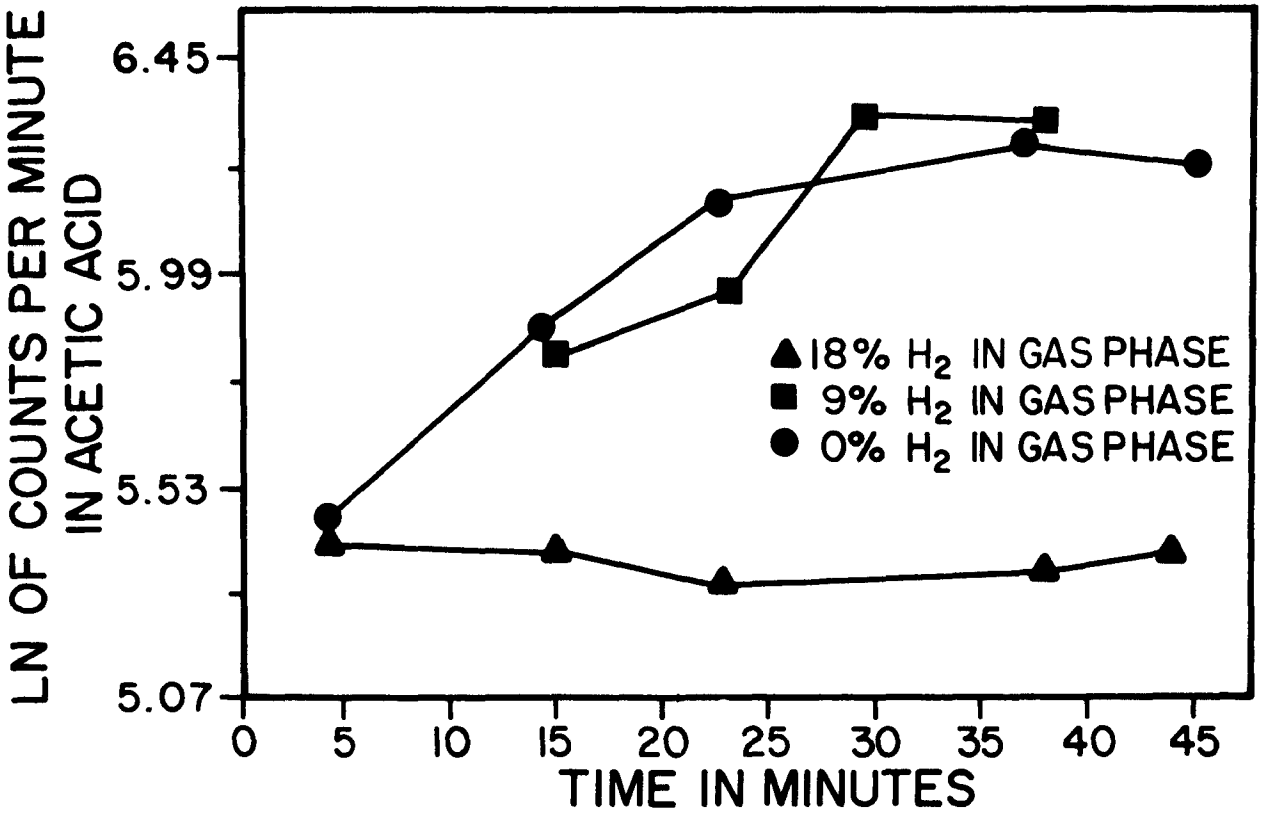
LN OF COUNTS PER MINUTE IN ACETIC ACID
6.45
5.99
5.53
5.07
0
5
10
15
20
25
30
35
40
45
TIME IN MINUTES
18% H_2 IN GAS PHASE
9% H_2 IN GAS PHASE
0% H_2 IN GAS PHASE

TABLE 5. EFFECT OF HYDROGEN GAS ON SLUDGE METHANOGENESIS

Treatment Prior to Inoculation	CH_4 Formed in μmole	Rate of CH_4 Formation in μmole/min
Exposed to H_2 gas*	62	0.017
" " " "	66	0.018
Exposed to N_2 gas+	75	0.020
" " " "	78	0.021

*Equilibrated with 70% H_2 and 30% CO_2 for two hours.
+Equilibrated with 70% N_2 and 30% CO_2 for two hours.
(Incubation time 75 minutes)

Hydrogen Uptake Capacity as a Parameter for Predicting Digester Failure

During the course of our studies it was observed that poorly digesting sludge had a limited capacity for hydrogen utilization. These observations suggested that hydrogen uptake by sludge might be a useful parameter for ascertaining digester functioning, and in the assay of effects of toxic substances on the digestion process. Long-term experiments were designed to provide information on the feasibility of these assays.

Two four-liter digesters were established using domestic sludge as feed. Both digesters were fed ten grams of solids per day with a hydraulic retention time of twenty days. Both digesters were then induced to fail. "Digester A" was induced to failure by maintaining the hydraulic retention time while the amount of solids fed was increased. "Digester B" was induced to failure by maintaining the amount of solids fed while the hydraulic retention time was decreased. The feeding schedule is shown in Figure 19.

Sludge samples were transferred to Warburg flasks and methane production was determined under an atmosphere of 70% N_2 and 30% CO_2, and under an atmosphere of 70% H_2 and 30% CO_2. Methane production from hydrogen-carbon dioxide atmosphere and total methane produced under a nitrogen-carbon dioxide atmosphere. The results are shown in Figures 20 and 21.

Volatile organic acid concentrations were determined for sludge from both digesters, with the results shown in Figures 22 to 26.

The changes in retention time and feeding rate were slow, producing slow changes in methane production during the initial stages of the experiment, as shown in Figures 20 and 21. Volatile organic acid concentrations increased as methane production decreased in both digesters. The organic acid concentration in digester A was quite different from the organic acid concentration in digester B at the time of failure. For these experiments failure was considered to have occurred when the sludge could not produce methane from hydrogen and carbon dioxide.

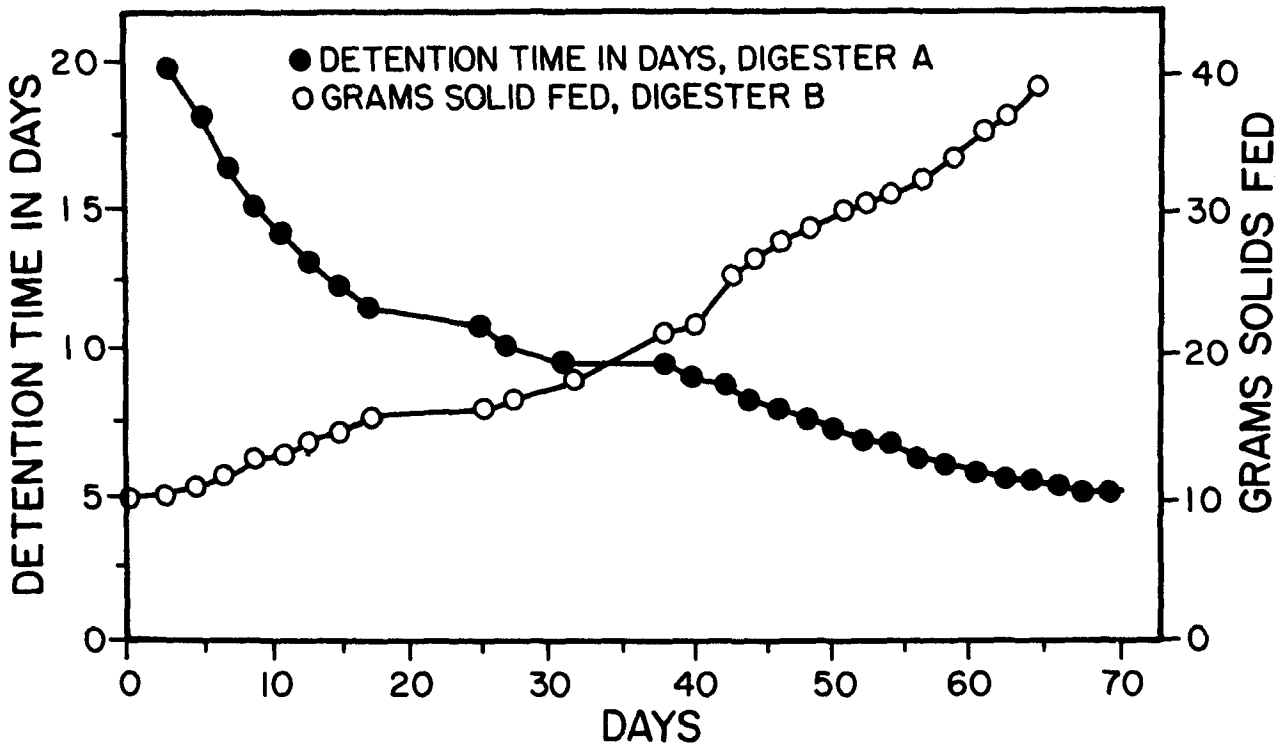

Figure 19. Feeding schedule of digesters being induced to fail.

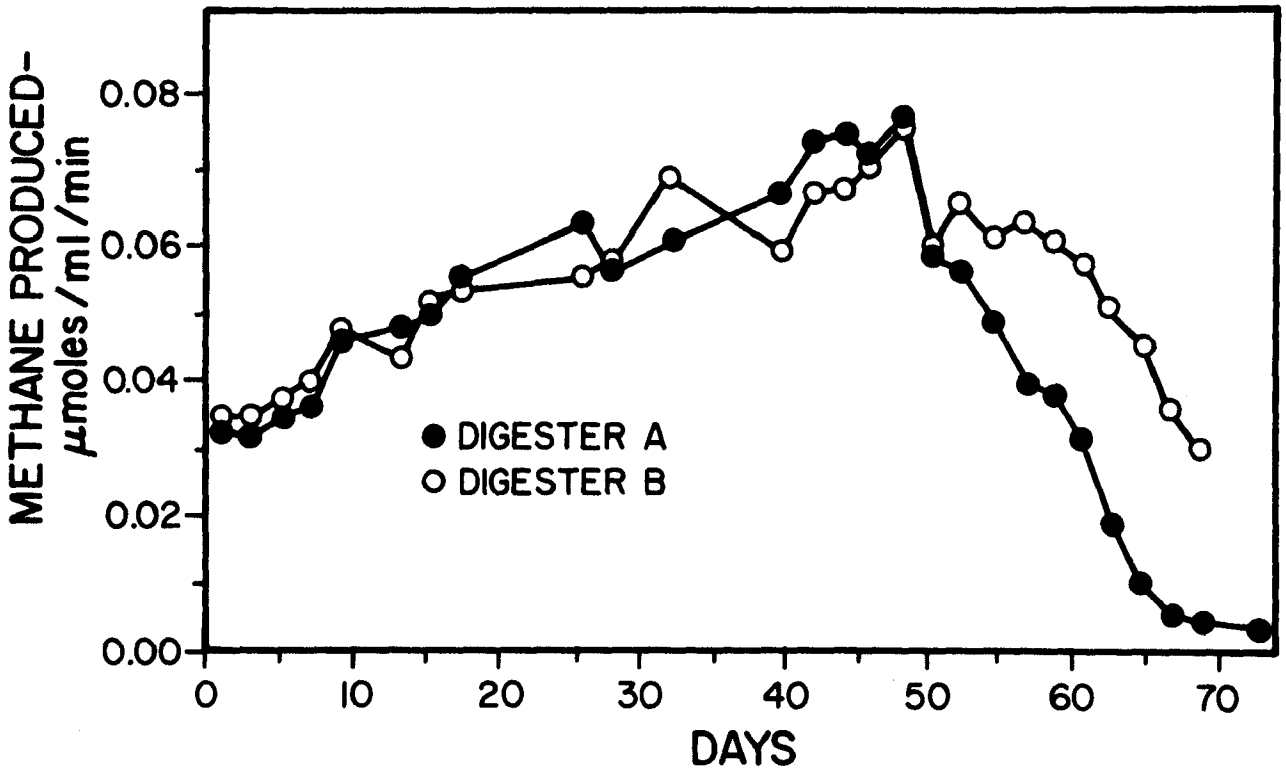

Figure 20. Methane production rates in digesters being induced to fail.

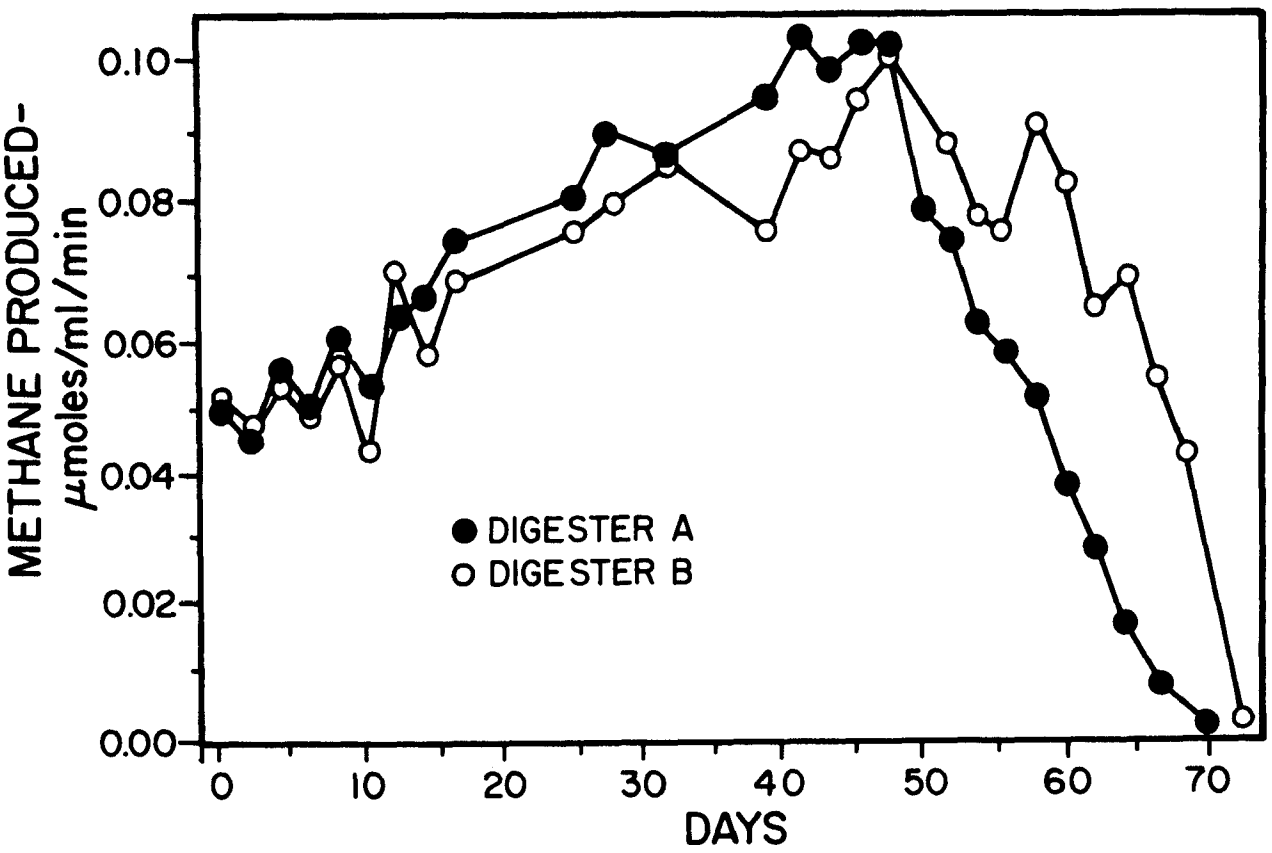

Figure 21. Methane production rates in digesters being induced to fail.

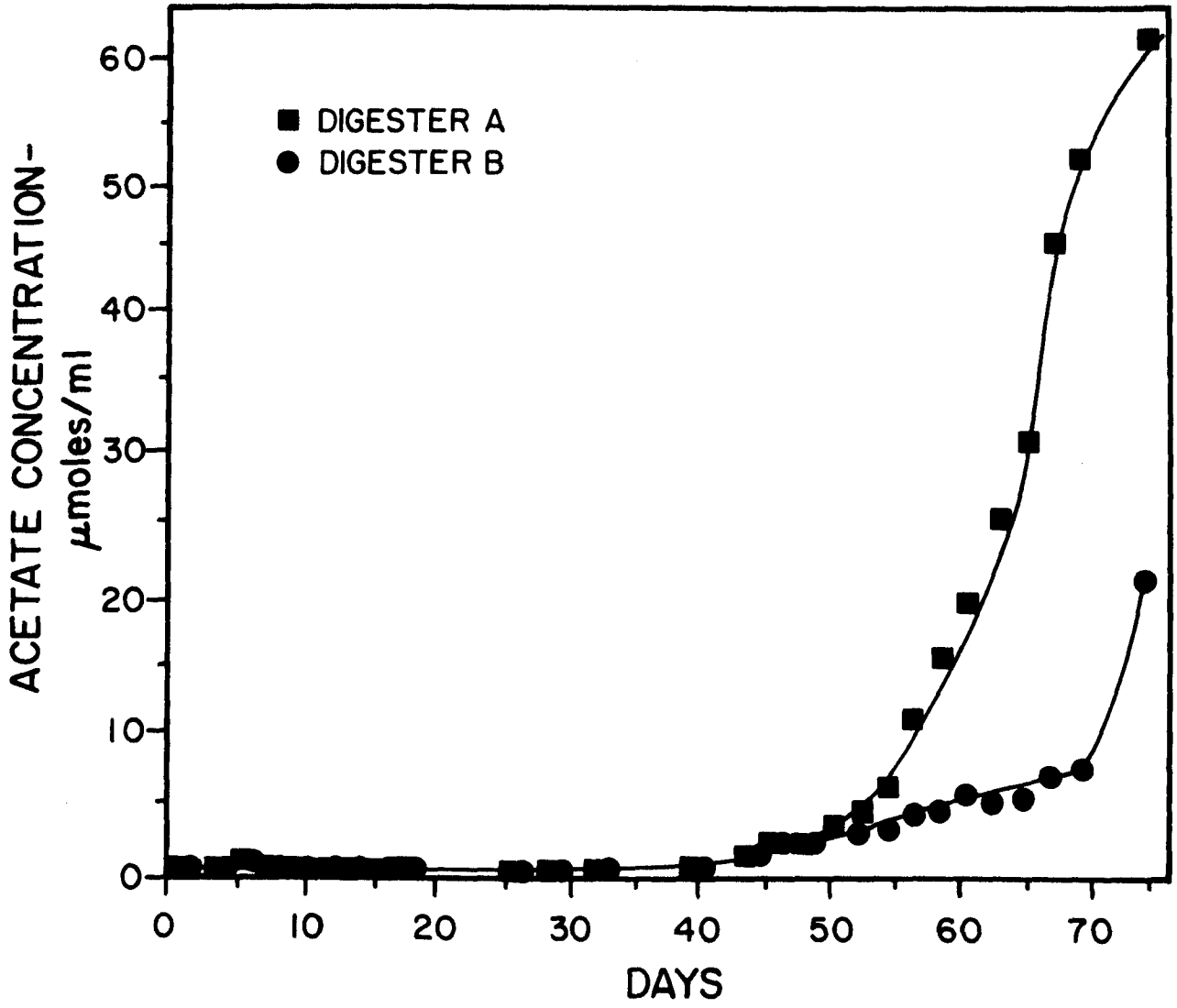

Figure 22. Acetate concentration in digesters being induced to fail.

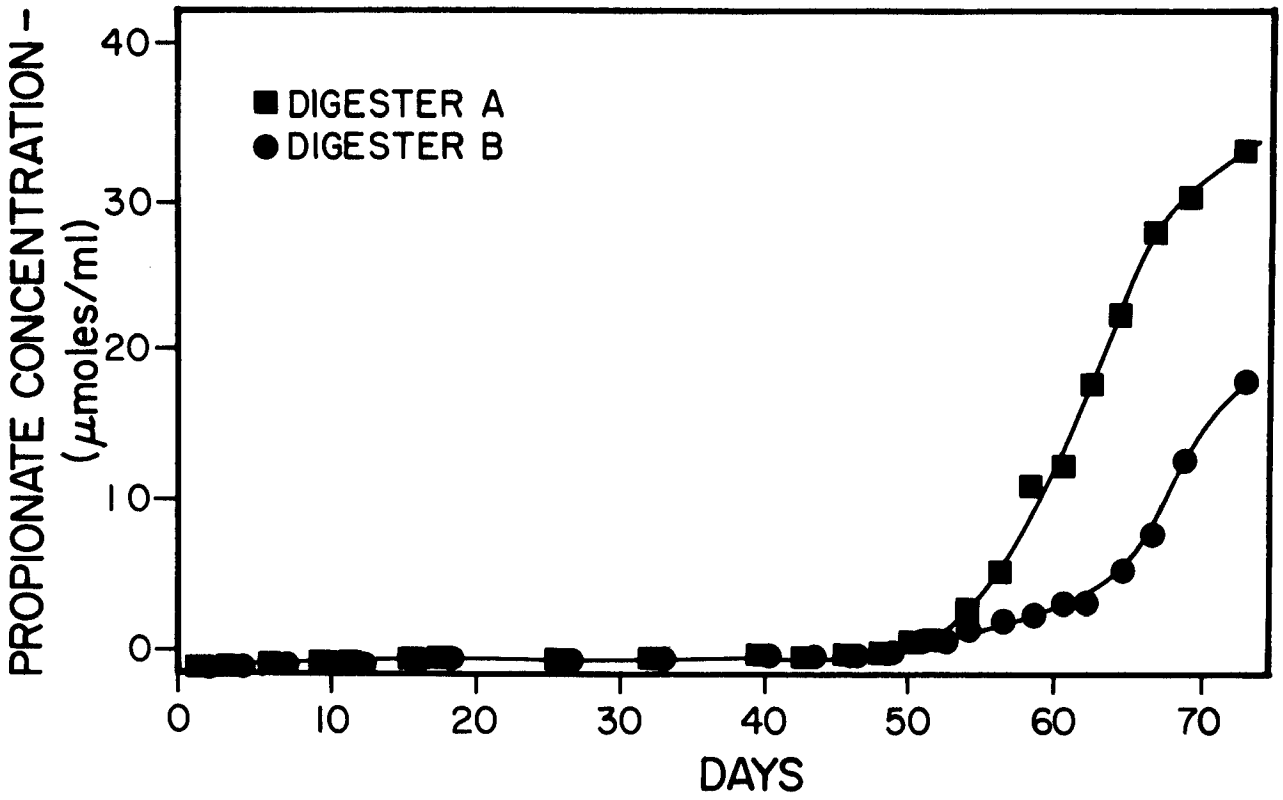

Figure 23. Propionate concentration in digesters being induced to fail.

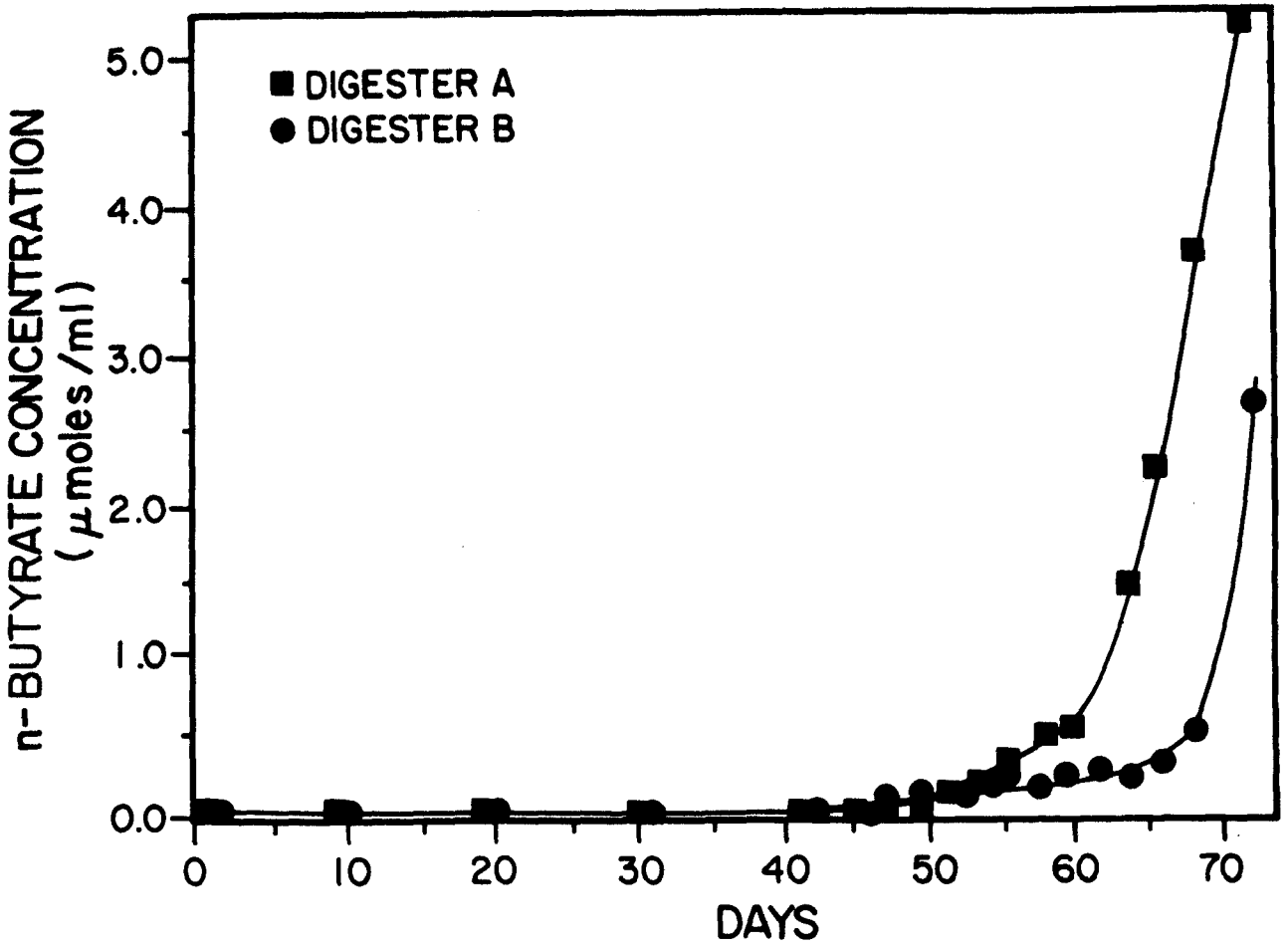

Figure 24. n-Butyrate concentration in digesters being induced to fail.

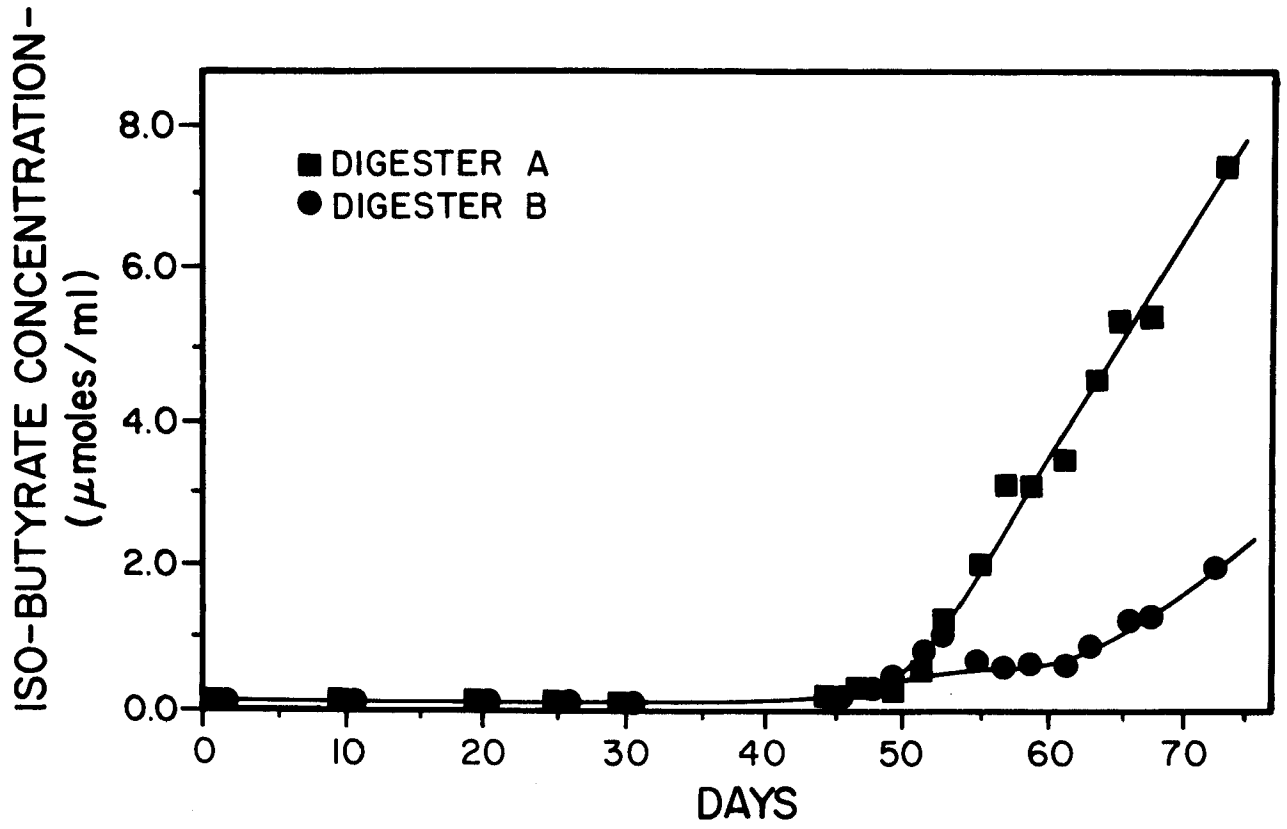

Figure 25. iso-Butyrate concentration in digesters being induced to fail.

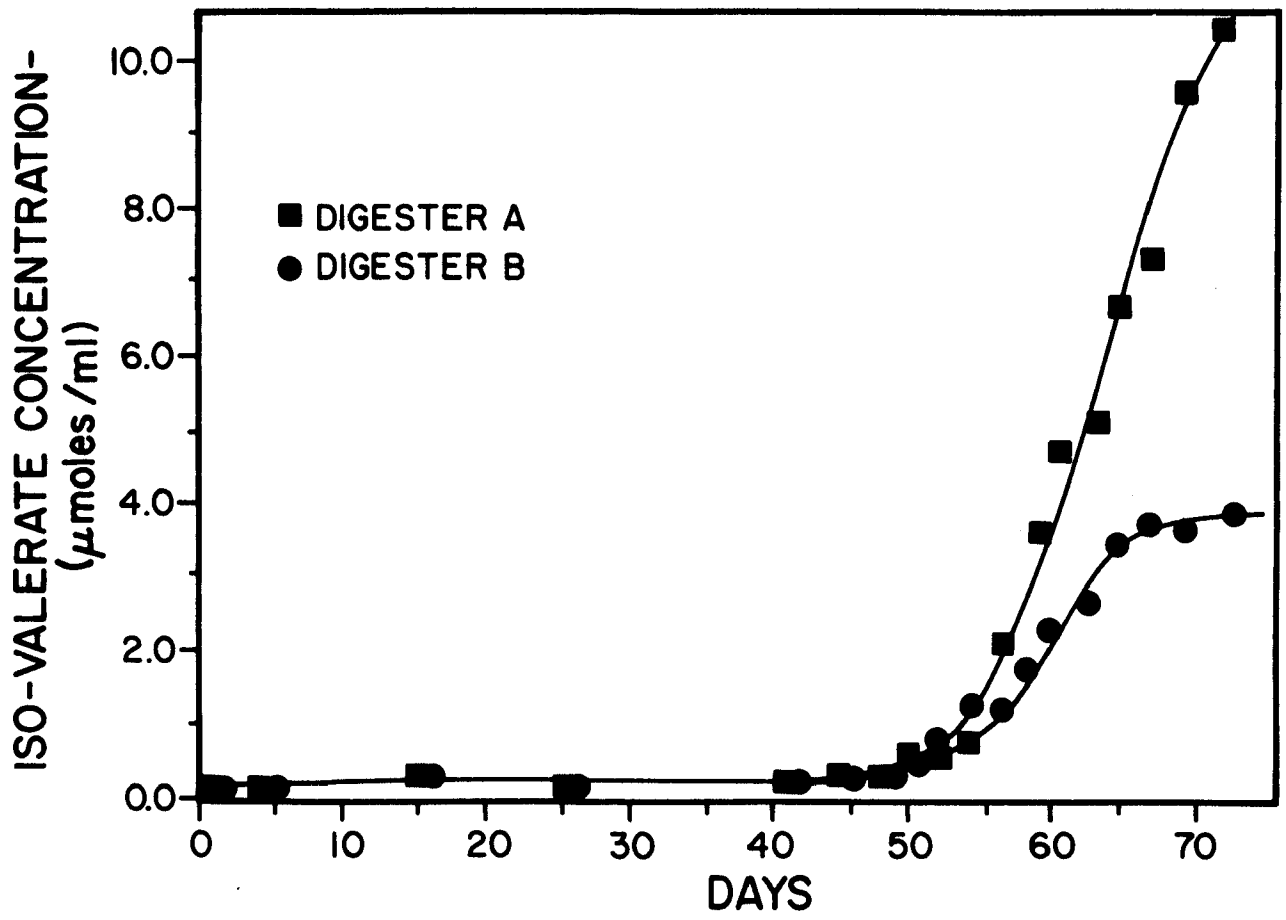

Figure 26. iso-Valerate concentration in digesters being induced to fail.

An effort was made to quantitate the relative contributions of acetate and propionate to methane production by a failing digester. Samples were analyzed when methane production was reduced to approximately 6% of the initial rate. Our attempts to obtain rate constants were unsuccessful as shown in Figures 27 and 28. Linearity could not be obtained.

The results obtained show that under the conditions imposed the capacity of sludge to produce methane from hydrogen and carbon dioxide reflects the capacity of sludge to produce methane from other sources. Due to the difficulty of the assay, and the fact that during failure decrease in methane production from hydrogen did not precede a decrease in methane formation from other sources, it is concluded that the assay of hydrogen conversion to methane is not a promising assay for predicting digester failure.

Digestion Inhibition Studies and Hydrogen Effects

We previously concluded that hydrogen gas specifically inhibited the metabolism of propionic acid in sludge. We continued these studies in the hopes of elucidating the roles of various physiological types of bacteria in the total fermentation. The initial studies dealt with short-term effects of hydrogen on the fermentation. These studies have been extended to include long-term effects with emphasis on the organic acids.

Small digesters were established and fed domestic sludge with a retention time of twenty days. Three experimental digesters were equilibrated with a gas phase of 70% hydrogen and 30% carbon dioxide. A fourth digester was equilibrated was a gas phase of 70% nitrogen and 30% carbon dioxide. Methane production and organic acid concentrations were followed with the results shown in Figures 29 to 31. Methane production from added hydrogen was determined by direct analysis of gases produced in the experimental digesters. Methane production from digesting sludge was determined in the experimental digesters by flushing out all hydrogen with a nitrogen-carbon dioxide mixture, and then directly assaying the gases produced.

The results showed that in the presence of hydrogen gas production of methane from digesting sludge was inhibited. The inhibition was very marked during the first twenty-four hours and was accompanied by a rapid increase in the concentration of propionic acid. On continued exposure to hydrogen, the propionic acid concentration continued to rise, followed after four days by an increase in concentration of acetic acid. After ten days exposure, methane production from the sludge was essentially zero. The small residual value shown is interpreted to have been formed from reserve materials in the hydrogen oxidizing microflora which developed on the added hydrogen.

From these, and our other reported results, we have concluded that the role of the hydrogen oxidizing methanogenic bacteria in anaerobic digestion is the maintenance of a hydrogen concentration low enough to prevent the inhibition of short chain organic acid metabolism and the cessation of the fermentation.

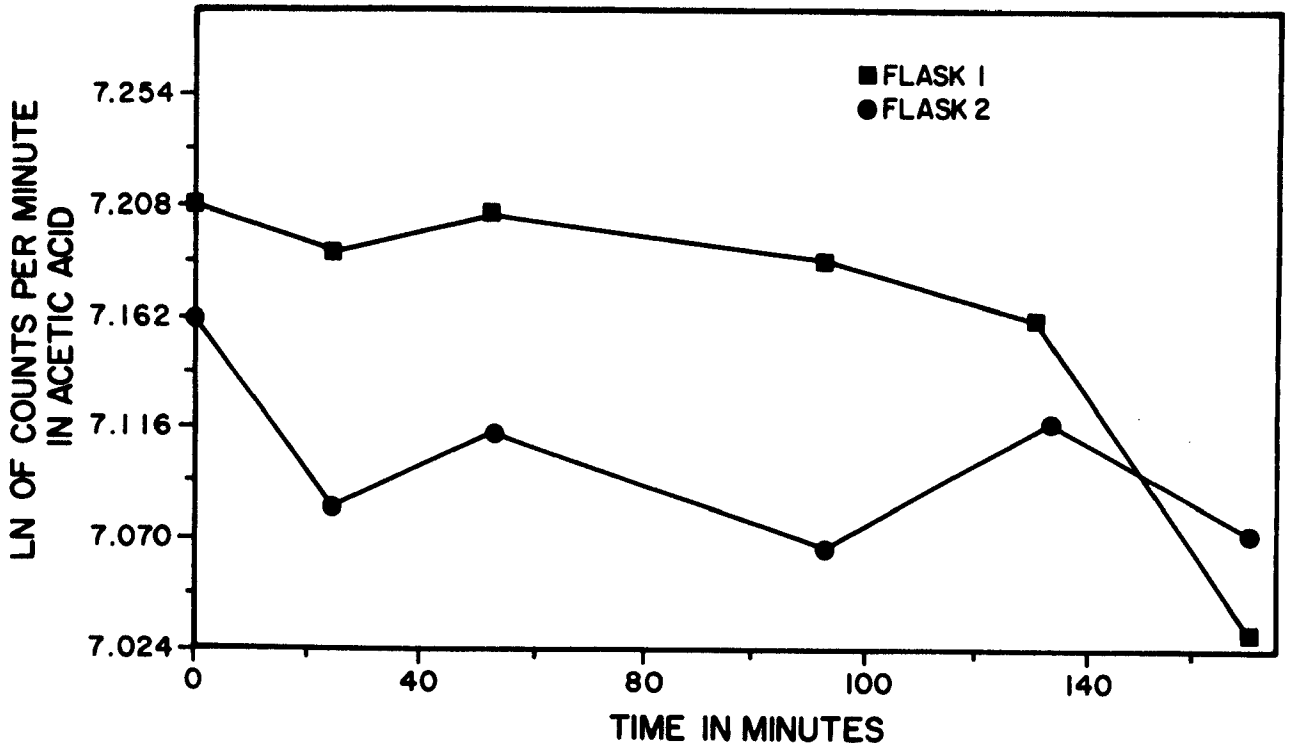

Figure 27. Change in the counts per minute in the acetate fraction from constant volumes of sludge following addition of radioactive acetate to digesters being induced to fail.

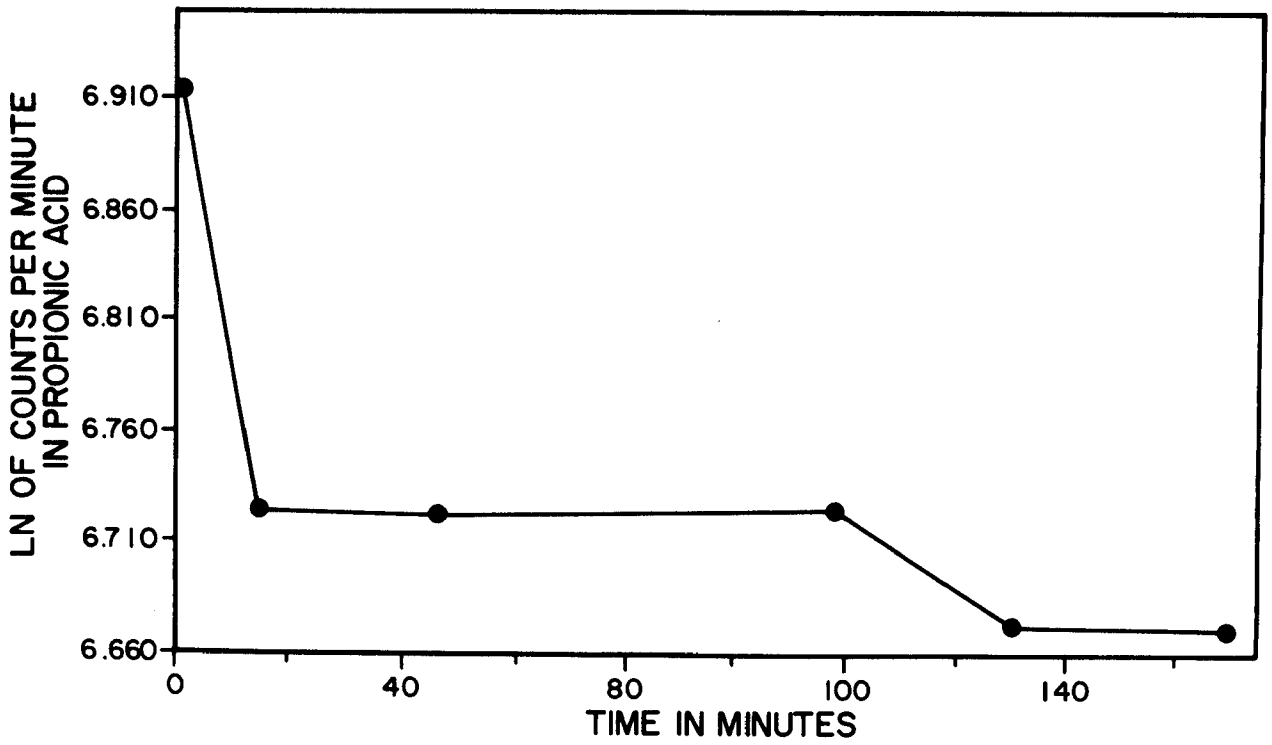

Figure 28. Change in the counts per minute in the propionate fraction from constant volumes of sludge following addition of radioactive propionate to digesters being induced to fail.

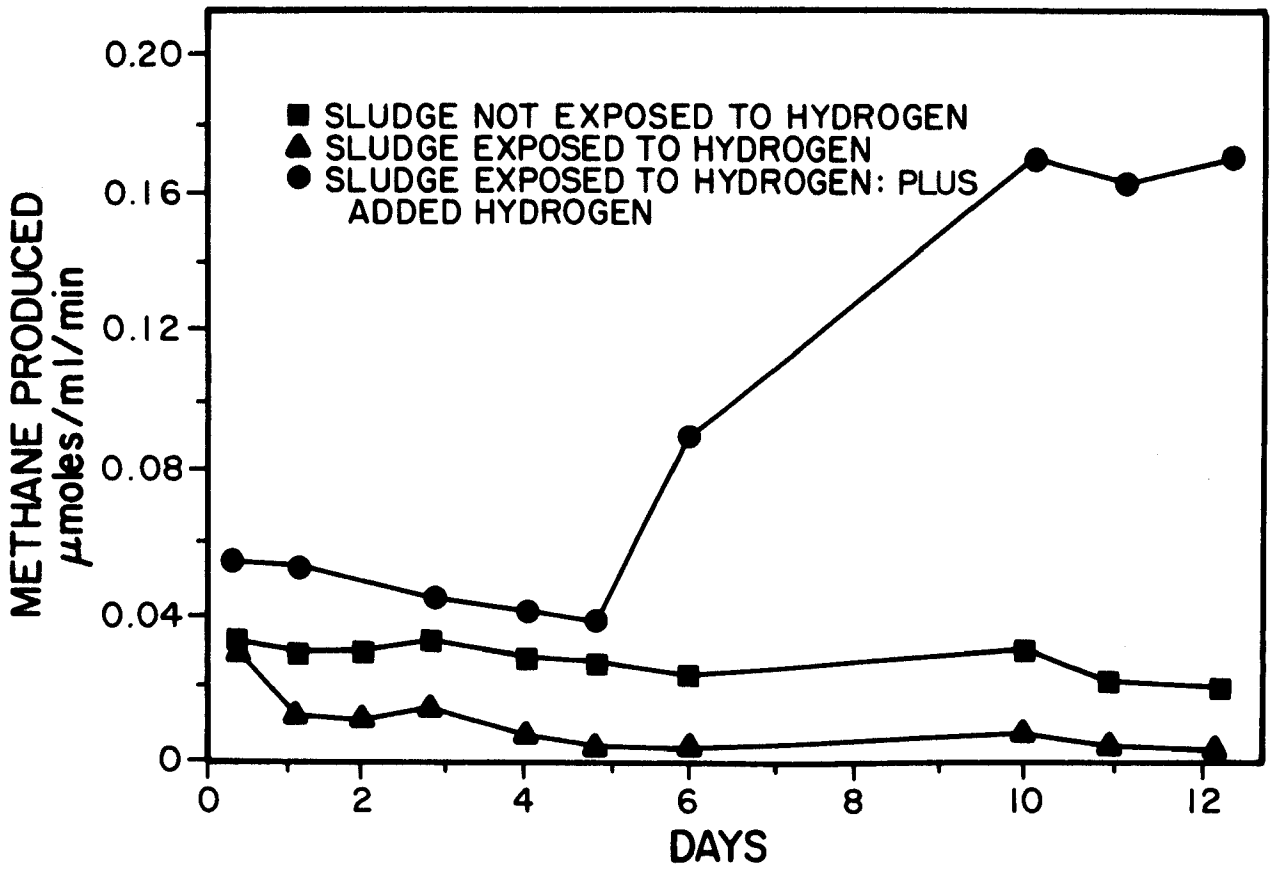

Figure 29. Methane production from sludge not exposed to hydrogen, sludge exposed to hydrogen and sludge exposed to hydrogen with added hydrogen as substrate.

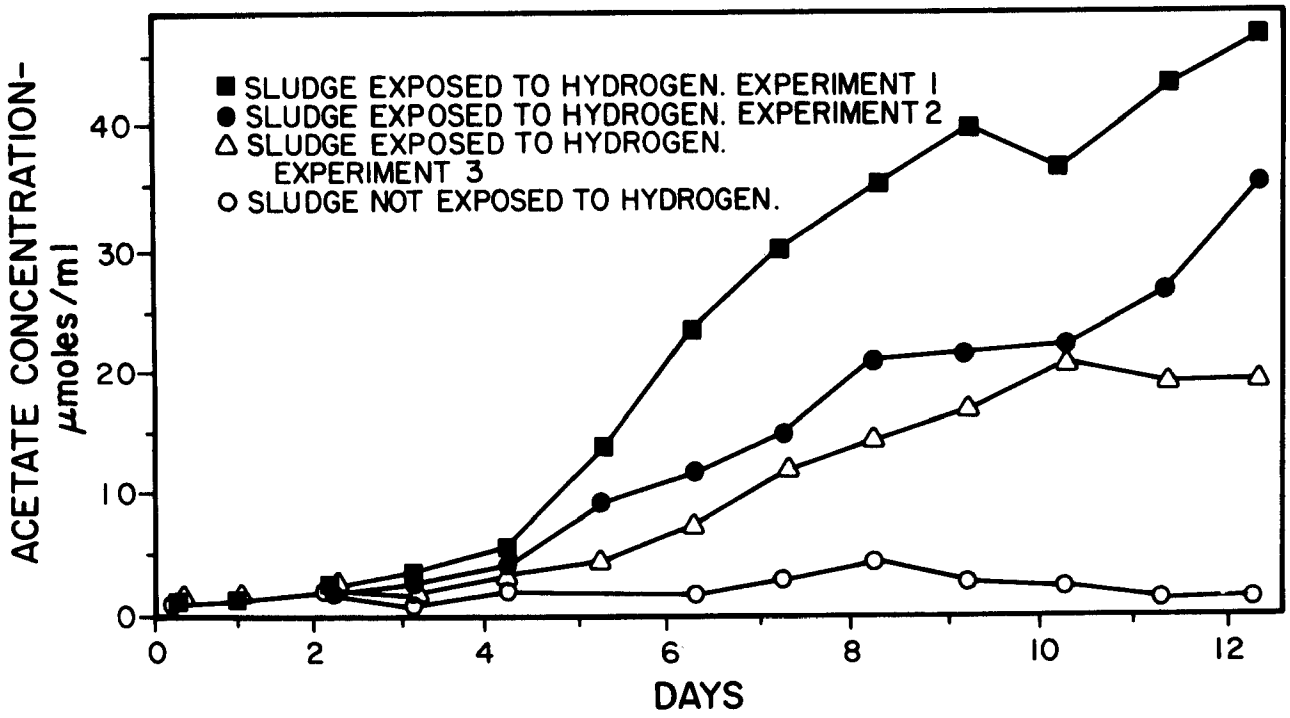

Figure 30. Acetate concentration in sludge exposed to hydrogen, and sludge not exposed to hydrogen.

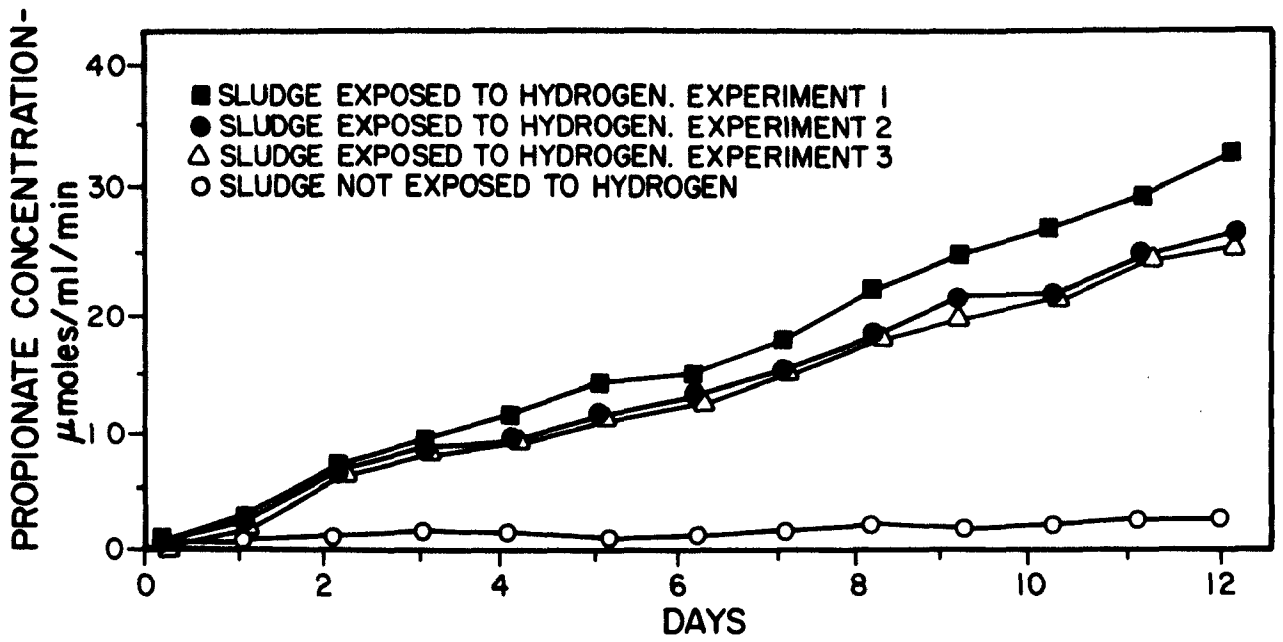

Figure 31. Propionate concentration in sludge exposed to hydrogen, and sludge not exposed to hydrogen.

Long-Term Hydrogen Inhibition

The previous experiments were conducted for twelve days. The experiments were then repeated over a sixty-five day period to determine which physiological groups of bacteria survived this form of inhibition. The experiments were conducted with duplicate samples. Figure 32 shows the results of hydrogen exposure over a 65-day period. Figures 33 and 34 show the rates of methane production from added hydrogen by sludge which had been exposed to hydrogen. Figures 35 and 36 show the acetate pools in sludge exposed to hydrogen. Figures 37 and 38 show the propionate pool sizes of sludge exposed to hydrogen. Figures 39 and 40 show the n-butyrate and the n-valerate pools of sludge exposed to hydrogen. Figures 41 and 42 show the iso-butyrate and iso-valerate pool sizes in sludge exposed to hydrogen.

These results show that hydrogen inhibits the dissimilation of propionate, n-butyrate, iso-butyrate, n-valerate and iso-valerate. Acetate is inhibited, assuming no formation of acetate from hydrogen and carbon dioxide, after about five days but after approximately twelve days the inhibition is reversed and the acetate pool size decreases rapidly. The results show that the inhibition by hydrogen affects a different microflora than the inhibition caused by reduced retention time, or increased feed rate, since in the former case the sludge retains the ability to produce methane from hydrogen and carbon dioxide, and acetate inhibition is temporary.

HYDROGEN PRODUCTION FROM VOLATILE ACIDS

Radioactive tracer studies (10, 19) of methanogenic enrichments and the data reported here have shown that acetate is an intermediate in the methanogenic dissimilation of propionate and butyrate. Using highly purified cultures which were unable to dissimilate acetate, Stadtman and Barker (20) demonstrated that in the presence of $H^{14}CO_3^-$, the methane produced from propionate and butyrate had the same specific activity as bicarbonate, indicating that all methane was produced by carbon dioxide reduction. These reactions can therefore be rewritten as the sum of an oxidation reaction and a reduction (methanogenic) reaction (Table 6, equations A through D).

The highly purified cultures used by Stadtman and Barker are generally believed to have been impure cultures (3) but direct evidence is lacking. No demonstrably pure methanogenic cultures have been shown capable of dissimilating either propionate or butyrate. The inability to use these substrates directly suggests that non-methanogenic bacteria may oxidize propionate and butyrate and provide hydrogen to methanogenic bacteria (4). Because molecular hydrogen is the preferred substrate for carbon dioxide reduction by methanogens (26), hydrogen is a likely candidate for a role in interspecies electron carrier. If this were the case, the dissimilation of propionate and butyrate could be written as the sum of a hydrogen producing and hydrogen utilizing reaction (Table 6, equations E through H). It can be seen from the free energy changes of equations E and G (Table 6) that the partial pressure of hydrogen must be maintained at a very low level to allow exergonic hydrogen production from propionate and butyrate. This could presumably be accomplished by the

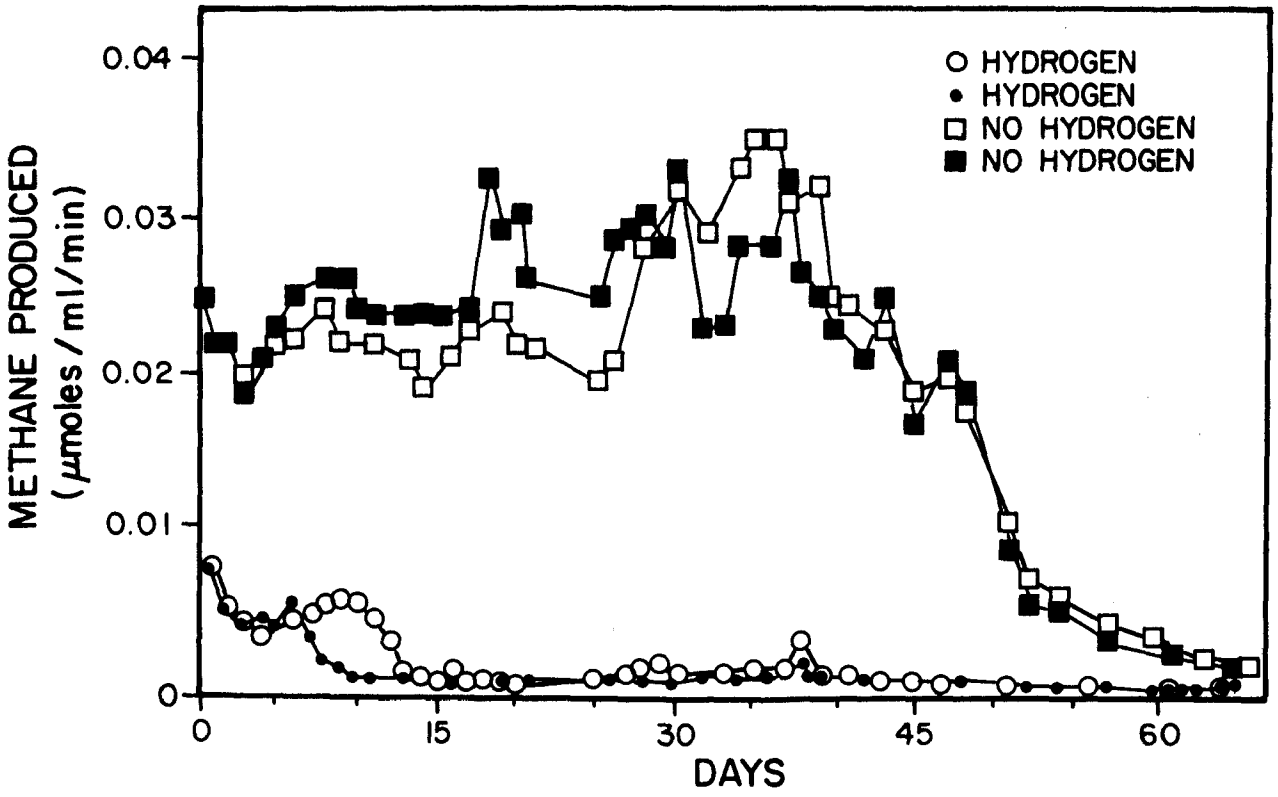

Figure 32. Methane production from sludge exposed to hydrogen, and sludge not exposed to hydrogen.

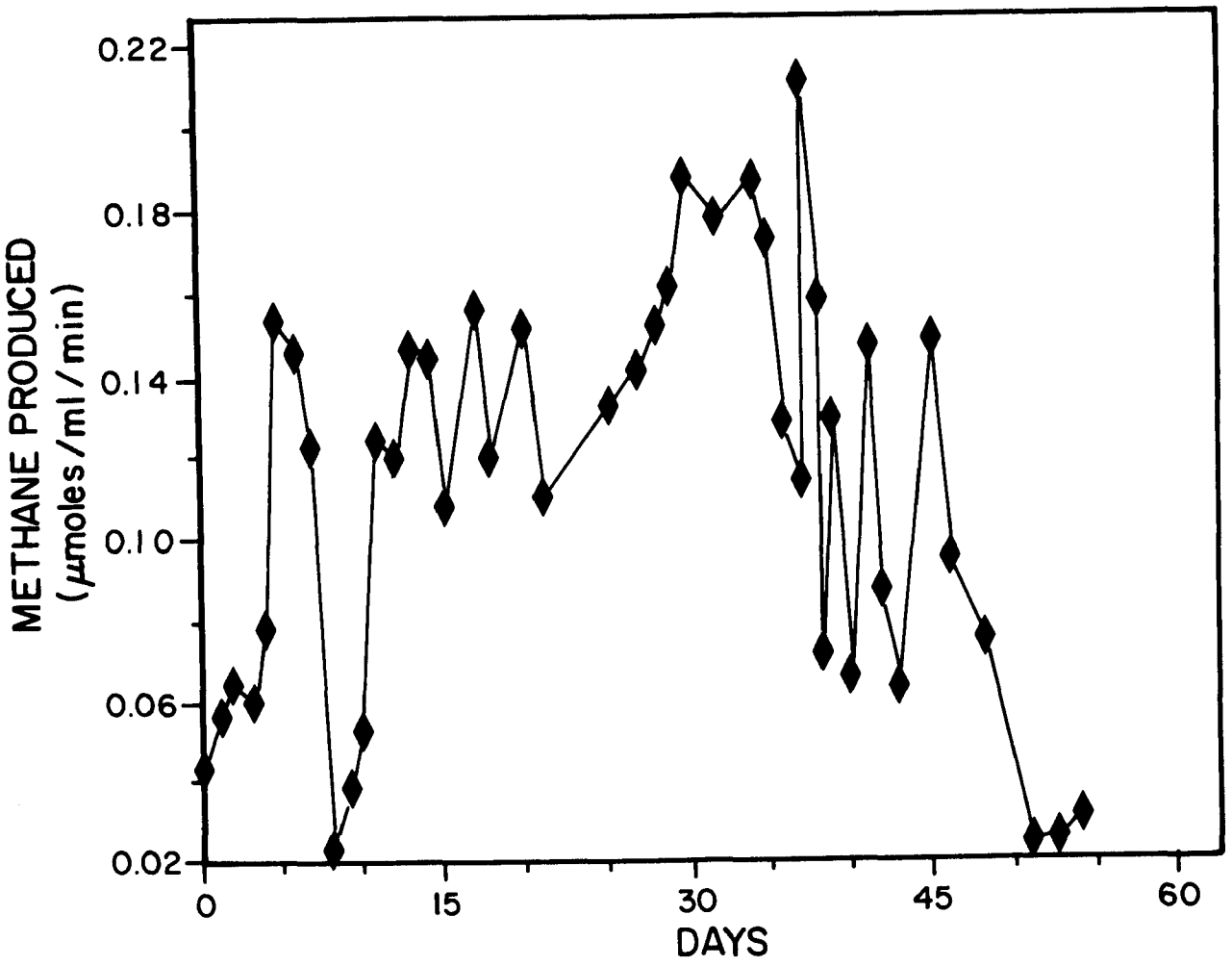

Figure 33. Methane production from added hydrogen by sludge previously exposed to hydrogen for periods of time up to 55 days.

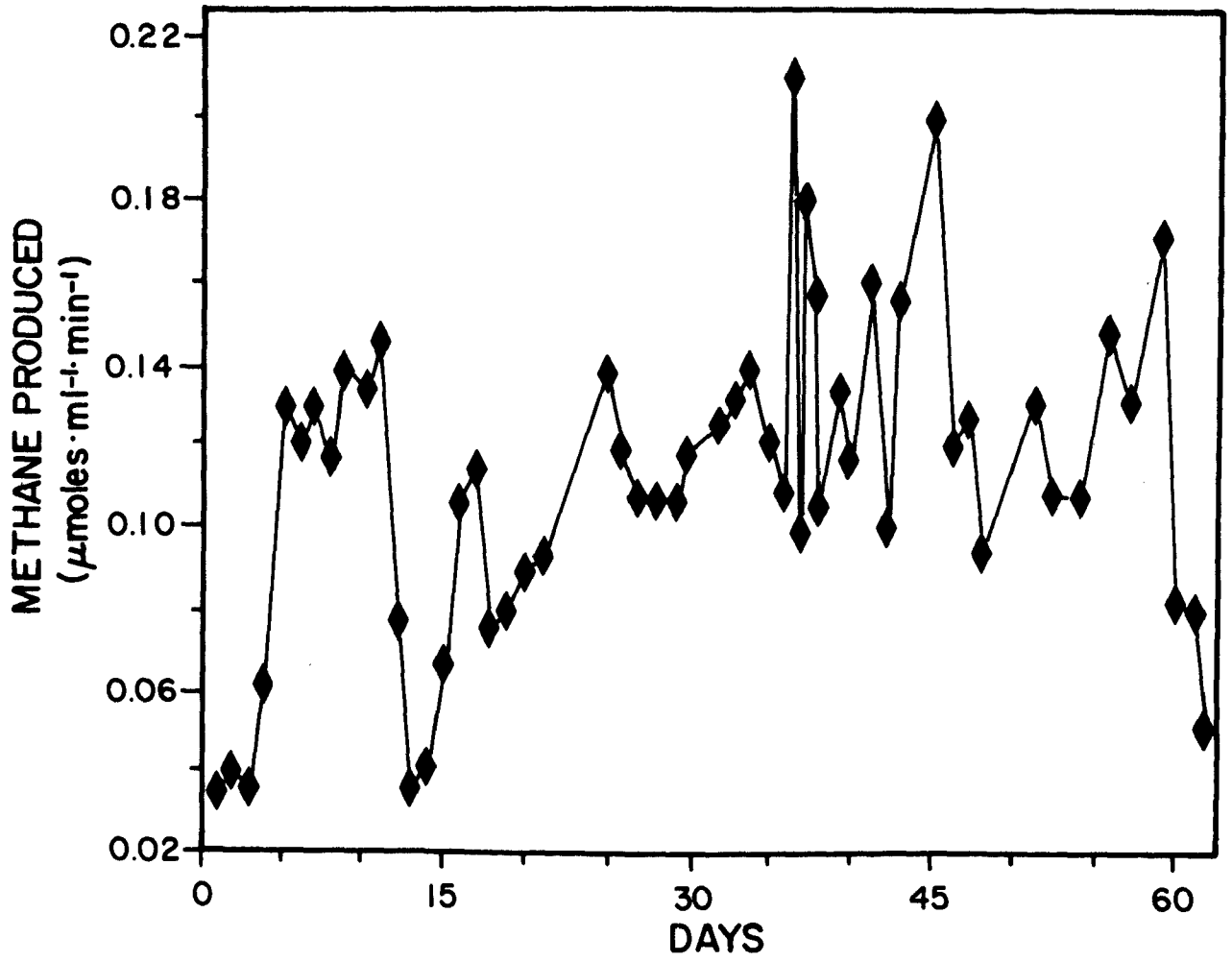

Figure 34. Methane production from added hydrogen by sludge previously exposed to hydrogen for periods of time up to 55 days.

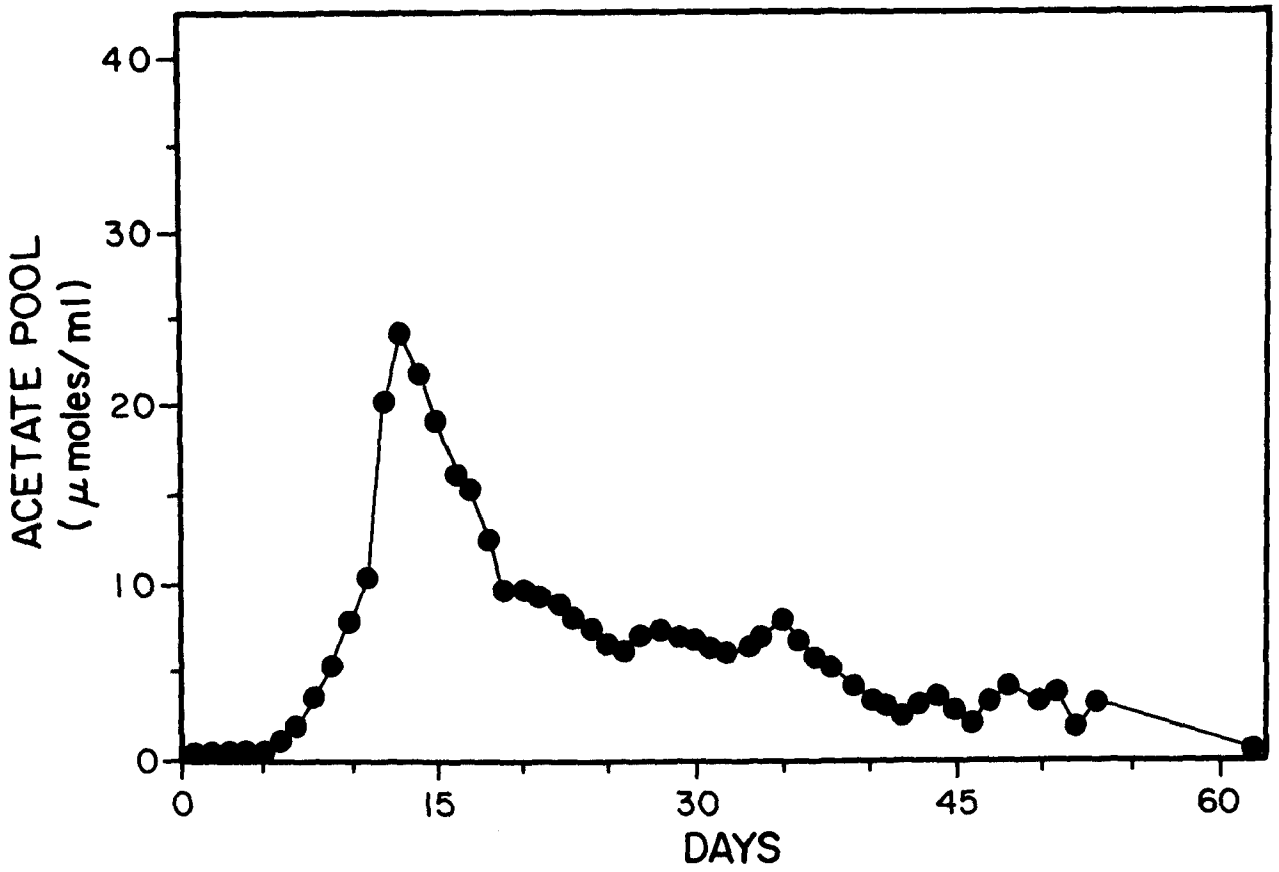

Figure 35. Acetate pools in sludge exposed to hydrogen for periods of time up to 55 days.

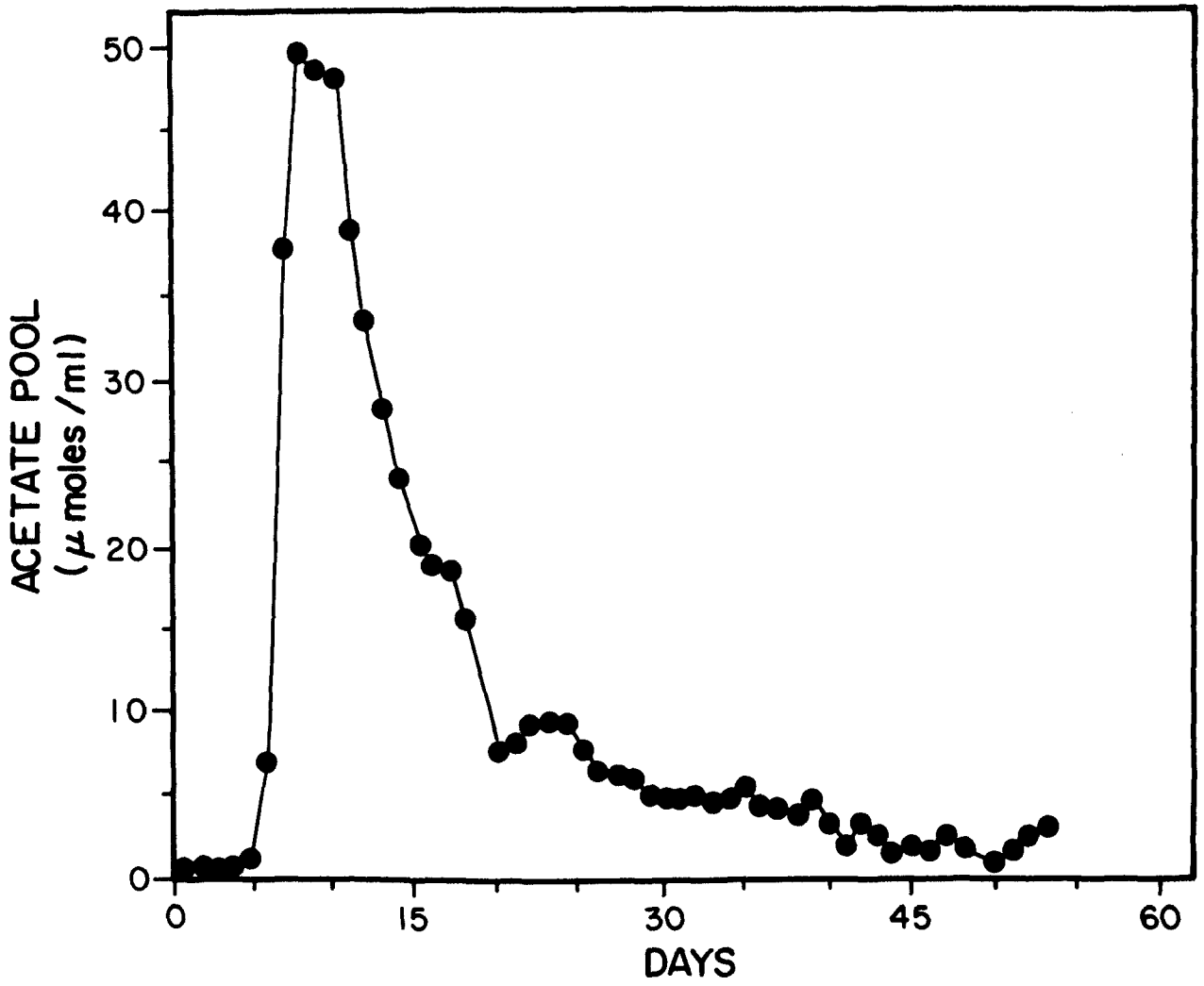

Figure 36. Acetate pools in sludge exposed to hydrogen for periods of time up to 55 days.

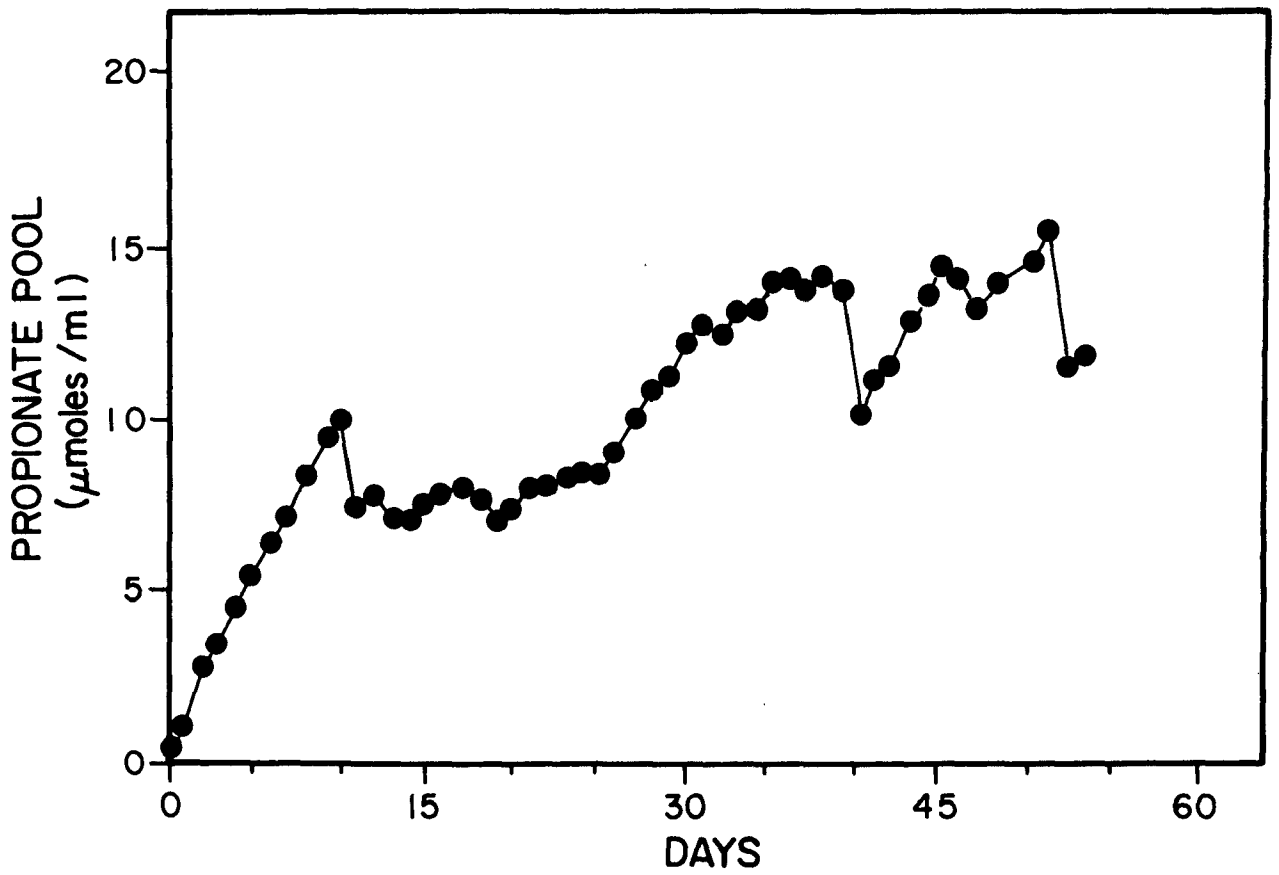

Figure 37. Propionate pools in sludge exposed to hydrogen for periods of time up to 55 days.

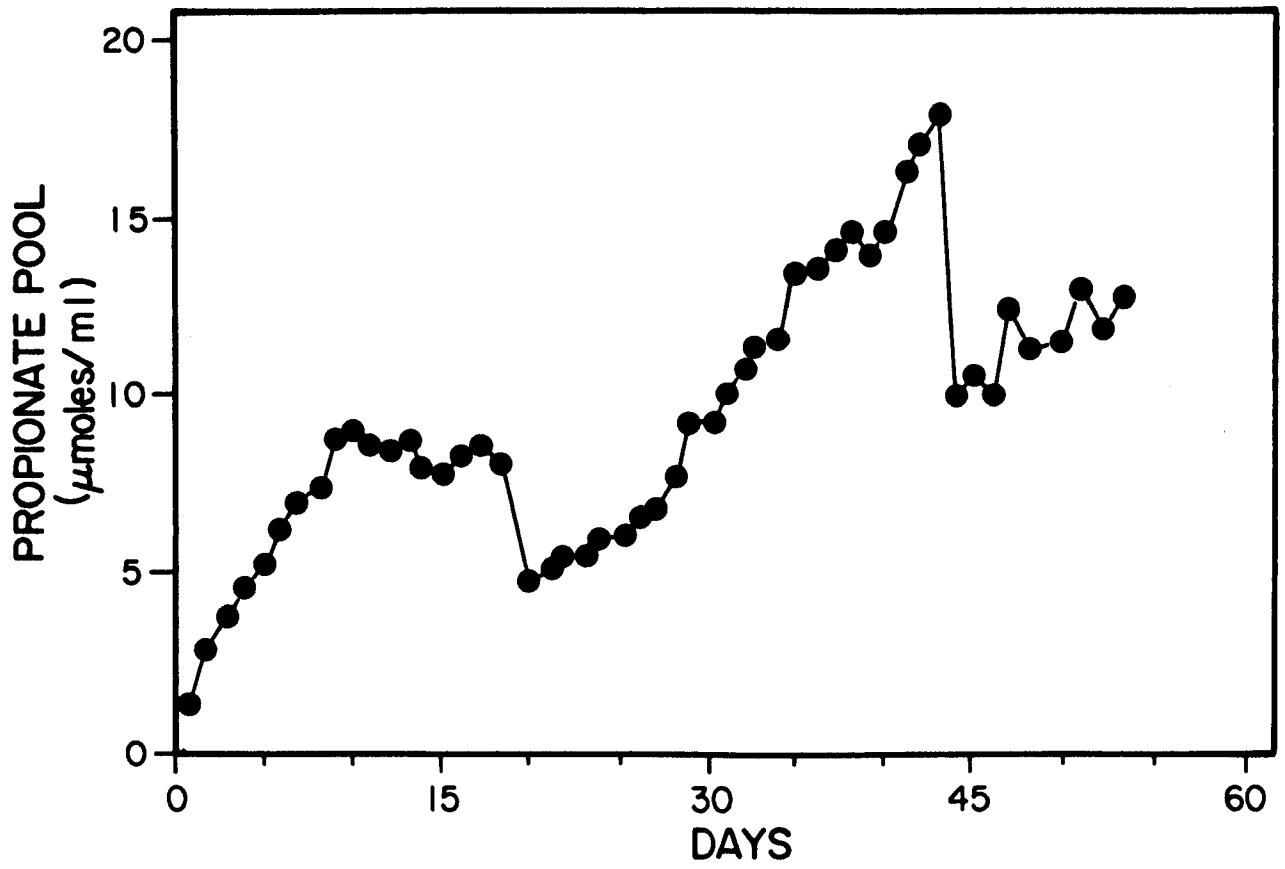

Figure 38. Propionate pools in sludge exposed to hydrogen for periods of time up to 55 days.

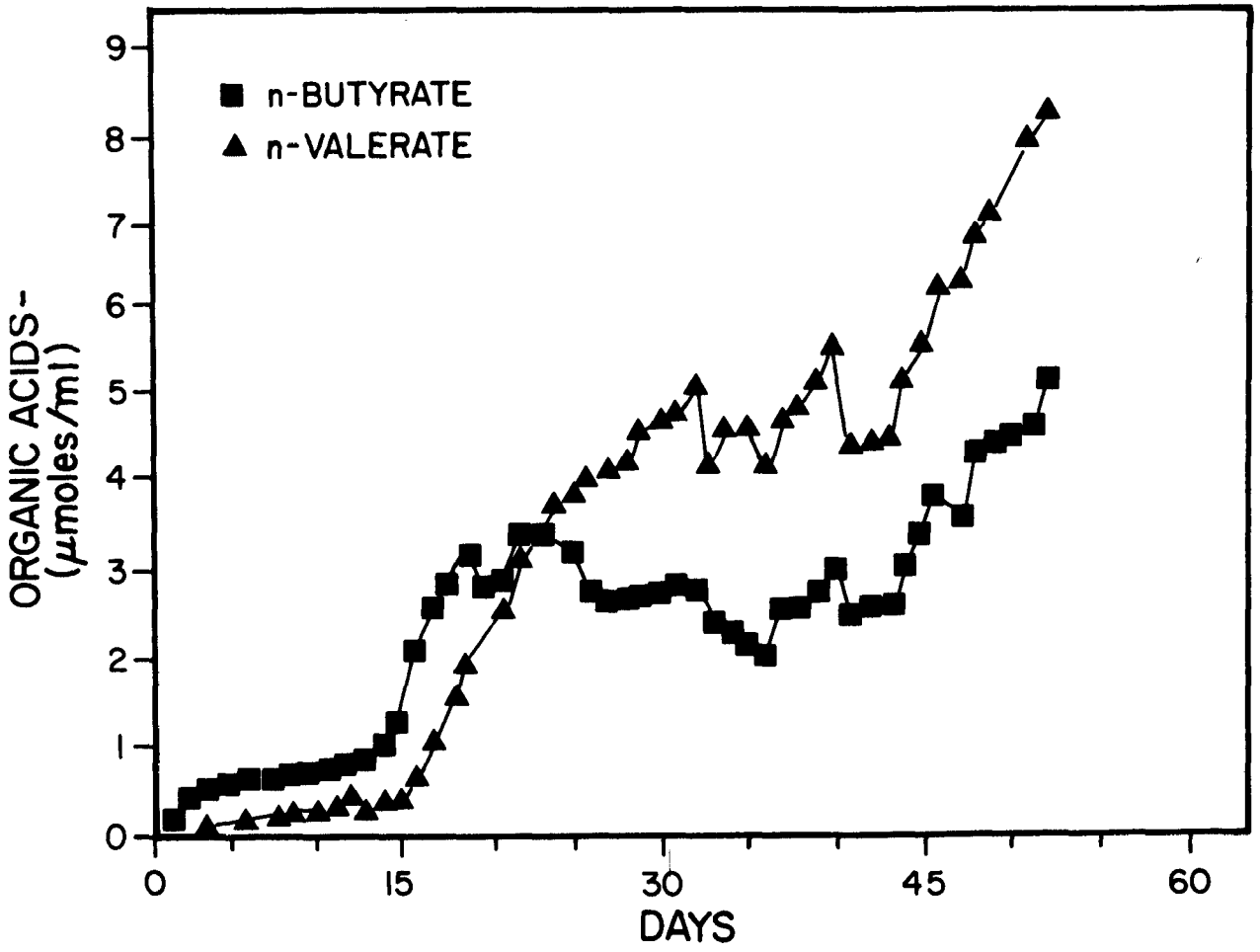

Figure 39. n-Butyrate and n-valerate pools in sludge exposed to hydrogen for periods of time up to 55 days.

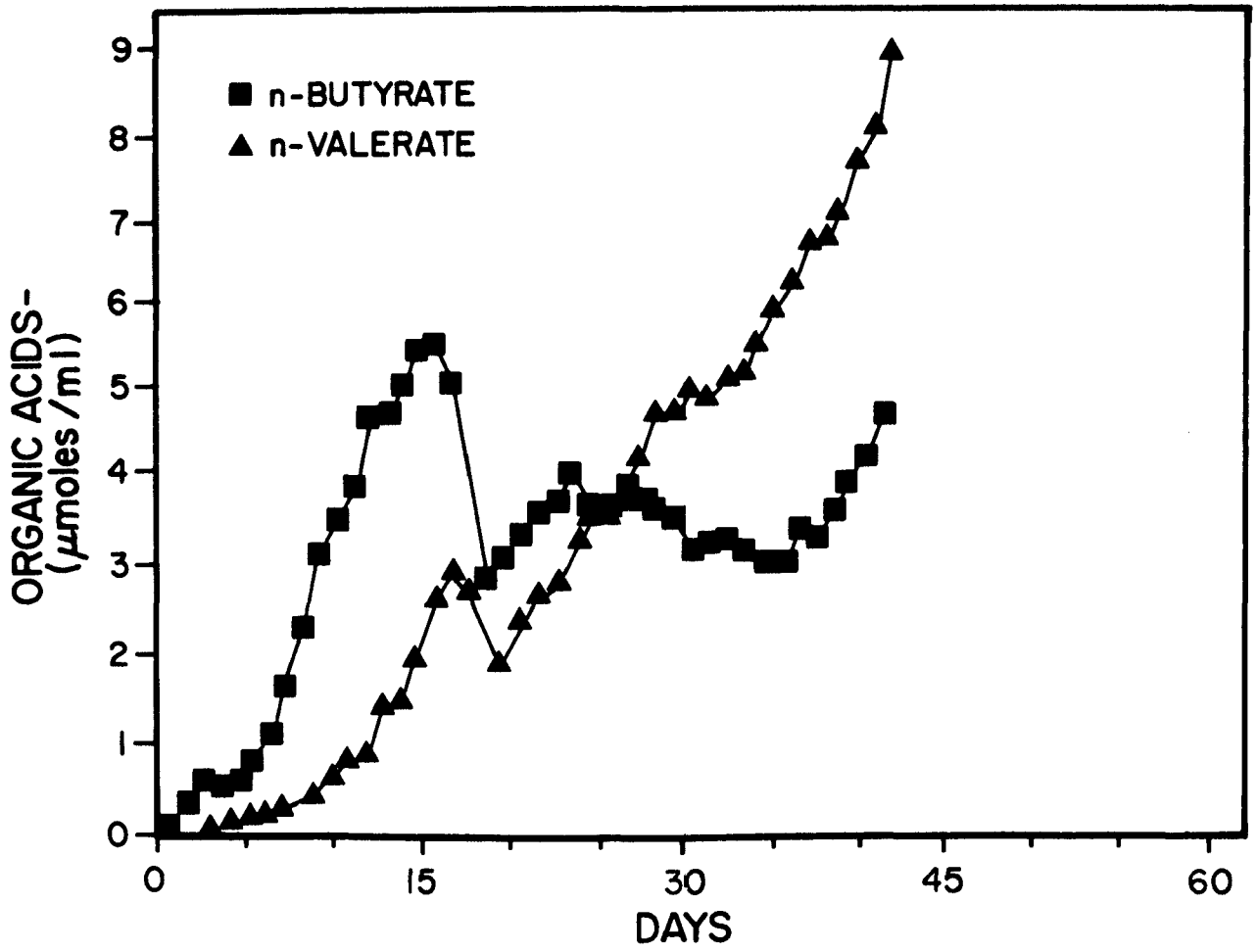

Figure 40. n-Butyrate and n-valerate pools in sludge exposed to hydrogen for periods of time up to 55 days.

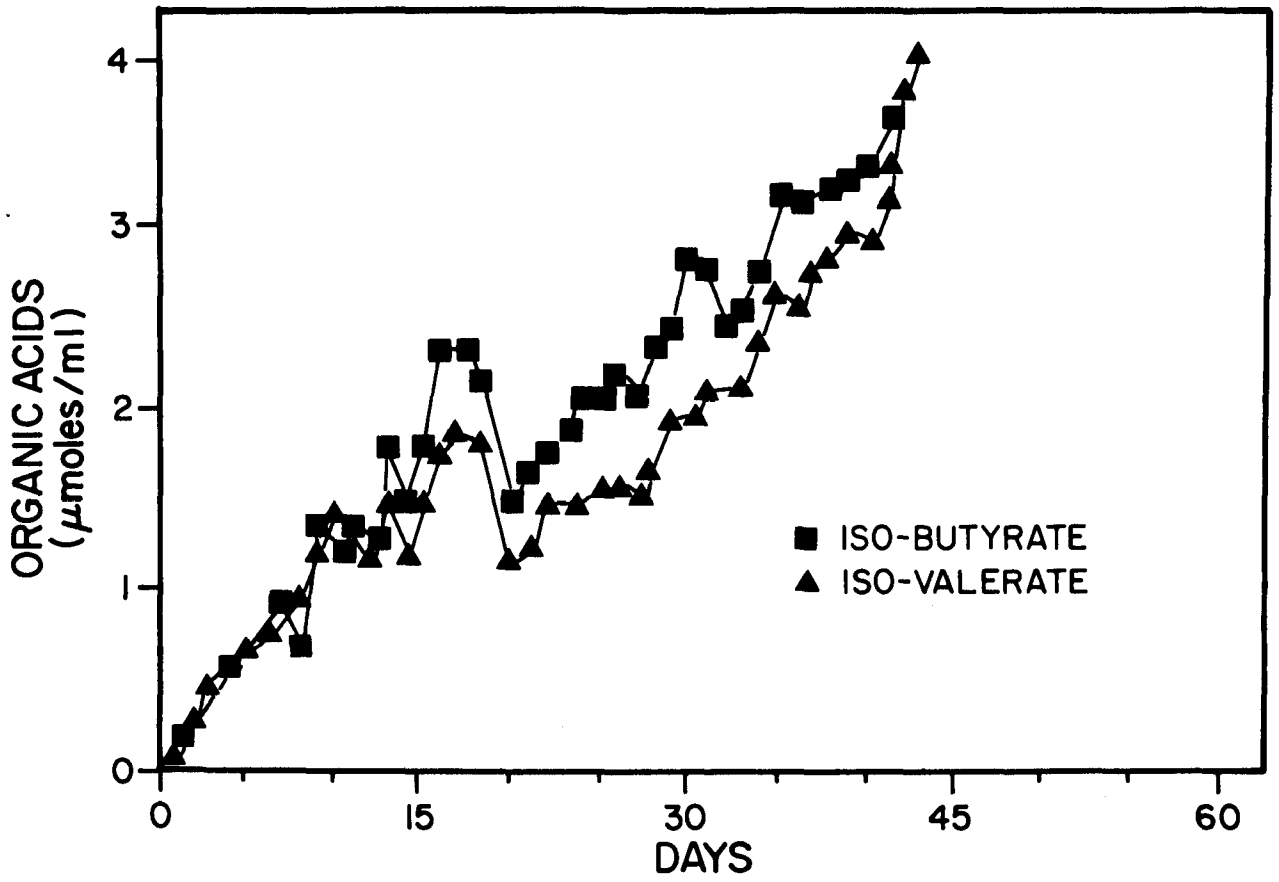

Figure 41. iso-Butyrate and iso-valerate pools in sludge exposed to hydrogen for periods of time up to 55 days.

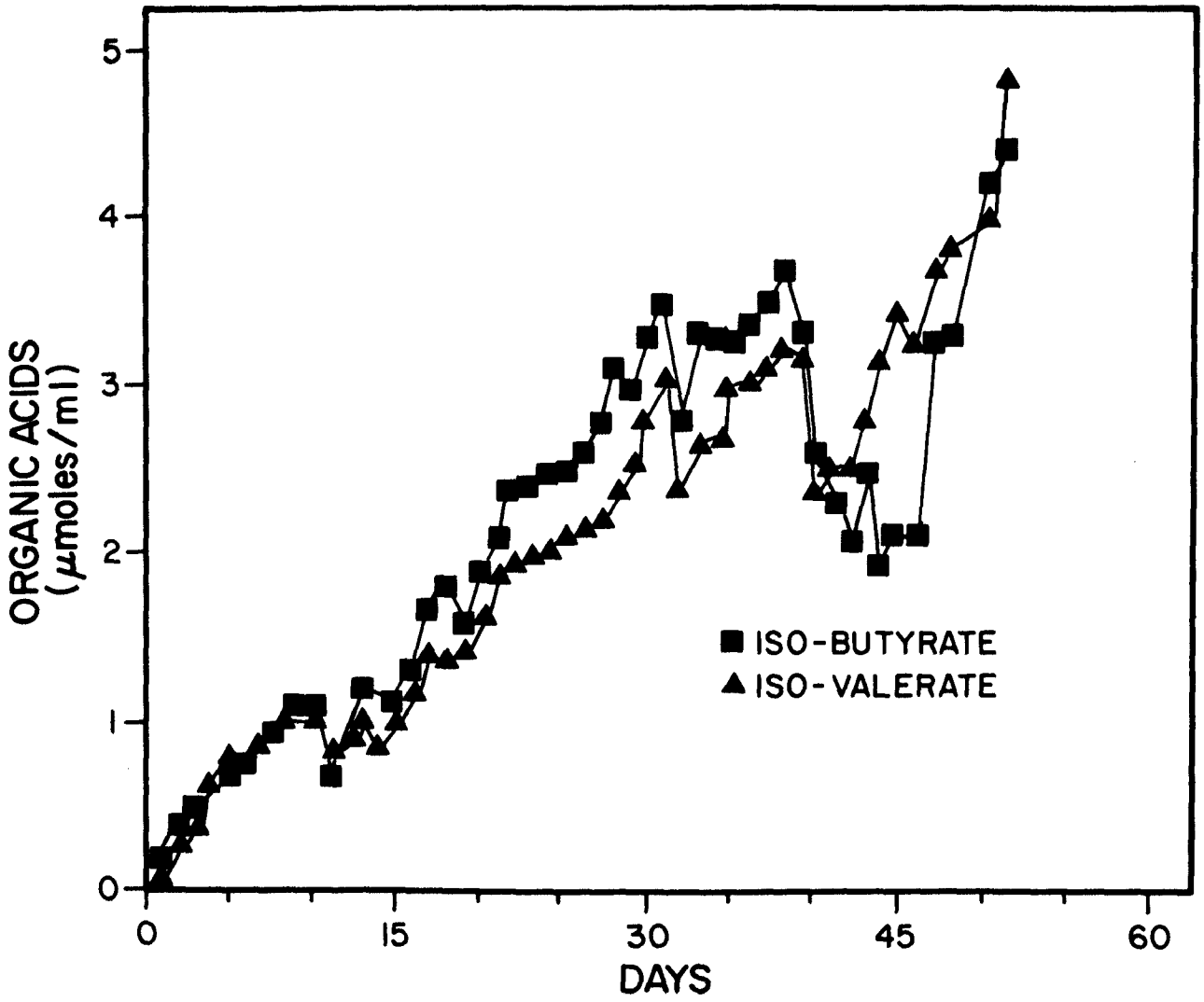

Figure 42. iso-Butyrate and iso-valerate pools in sludge exposed to hydrogen for periods of time up to 55 days.

TABLE 6. EQUATIONS AND FREE ENERGY CHANGES FOR THE ANAEROBIC OXIDATION OF PROPIONATE AND BUTYRATE, AND THE REDUCTION OF CO_2 TO METHANE

	Equation	$\Delta G^{\circ\prime}$ (kcal/react.)[a]	ΔG (kcal/react.)[b]
A	$4CH_3CH_2COO^- + 8H_2O \rightarrow 4CH_3COO^- + 4CO_2 + 24H^+ + 24\ e^-$	--	--
B	$24e^- + 24\ H^+ + 3CO_2 \rightarrow 3CH_4 + 6\ H_2O$	--	--
A + B	$4CH_3CH_2COO^- + 2H_2O \rightarrow 4CH_3COO^- + CO_2 + 3CH_4$	-25.23	-23.72
C	$2CH_3CH_2CH_2COO^- + 4H_2O \rightarrow 4CH_3COO^- + 8H^+ + 8e^-$	--	--
D	$8e^- + 8H^+ + CO_2 \rightarrow CH_4 + 2H_2O$	--	--
C + D	$2CH_3CH_2CH_2COO^- + 2H_2O + CO_2 \rightarrow 4CH_3COO^- + CH_4$	-8.16	-19.65
E	$4CH_3CH_2COO^- + 8H_2O \rightarrow 4CH_3COO^- + 4CO_2 + 12H_2$	+68.52	-1.86
F	$12H_2 + 3CO_2 \rightarrow 3CH_4 + 6H_2O$	-93.75	-21.86
E + F	$4CH_3CH_2COO^- + 2H_2O \rightarrow 4CH_3COO^- + CO_2 + 3CH_4$	-25.23	-23.72
G	$2CH_3CH_2CH_2COO^- + 4H_2O \rightarrow 4CH_3COO^- + 4H_2$	+23.09	-12.37
H	$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	-31.25	-7.28
G + H	$2CH_3CH_2CH_2COO^- + 2H_2O + CO_2 \rightarrow 4CH_3COO^- + CH_4$	-25.23	-23.72

a. H_2, CH_4, and CO_2 in gaseous state; H^+ at 10^{-7} mole activity per kg; all other substances at 1 mole activity per kg. The free-energy data have been calculated using free energies of formation from the elements compiled by Thauer et al. (16).

b. ΔG calculated at the following activities: Propionate, butyrate, and acetate at 10^{-3} moles/kg, CO_2 at 0.3 atm, CH_4 at 0.7 atm, and H_2 at 5×10^{-5} atm.

large numbers of hydrogen oxidizing methanogens present in methanogenic ecosystems (26).

Growth yield studies of hydrogen-grown methanogens indicate 2.3 to 3.3 g of cells per mole of methane produced (16, 18, 32), or (assuming y_{ATP} of 10.5 g/mole ATP) 0.21 to 0.31 moles ATP/mole CH_4. Thauer et al., (24) indicate that, including allowances for inefficiencies, 10 to 12 Kcal/mole are required for ATP production from ADP and inorganic phosphate. Taking a conservative view, allowing 12 Kcal/mole ATP and 0.31 moles ATP/mole CH_4, one can calculate that 3.72 Kcal/mole CH_4 must be coupled to ATP production. If one assumes that methane production from hydrogen and carbon dioxide approaches equilibrium in methanogenic ecosystems, one can substitute into the free energy equation for methane production according to equation H in Table 6:

$$\Delta G = \Delta G^\circ + RT \ln \frac{[CH_4]}{[CO_2][H_2]^4}$$

If one sets the partial pressure of CH_4 and CO_2 at values common to methanogenic ecosystem (.7 atm and .3 atm respectively), solving the equation for the partial pressure of hydrogen gives a value of 1.1×10^{-5} atm, well below the level necessary to allow exergonic hydrogen production from propionate or butyrate.

Interspecies transfer of electrons via molecular hydrogen has been demonstrated in a number of fermentative bacteria (5, 6, 9, 12, 14, 15, 17, 26, 29). When co-inoculated with methanogens, the soluble products of these fermentative bacteria are generally more oxidized than when grown in pure culture. Large amounts of hydrogen (measured as methane production) are the major reduced product when cocultured with methanogens, whereas little or no hydrogen is produced by the pure cultures. This phenomenon of interspecies hydrogen transfer has recently been reviewed by Mah et al. (13).

If interspecies hydrogen transfer is to occur, then there must be a hydrogenogenic microflora which produces hydrogen from volatile organic acids. The production of hydrogen from volatile acids is demonstrated by the experiments which follow. This unique form of hydrogen production was first reported by Smith and Shuba (23); however, in those experiments there was an inhibition of the reaction rates. The results could, therefore, be explained on the basis of a side reaction caused by the inhibition. In the experiments now reported the inhibition has been eliminated.

Determination of Inhibitory Concentrations of Volatile Acids

To insure that the levels of volatile organic acids used in subsequent experiments were not inhibitory, enrichments were incubated in the presence of various amounts of volatile organic acids, and the rate of gas production was monitored.

Warburg vessels were gased out with a gas mixture of 30% CO_2 - 70% N_2, and 50 ml of enrichment culture (acetate, propionate, or butyrate) were added

to vessels with varying amounts of volatile organic acids (oxygen-free solution neutralized with NaOH). Manometric readings were taken hourly, and at the end of each experiment liquid samples were taken for volatile organic acid analysis. Figure 43 illustrates that for these enrichment cultures, volatile organic acid concentrations up to 50 mM were not inhibitory. All subsequent experiments were performed with the volatile organic acid concentrations between 5 and 50 mM.

Effect of Molecular Hydrogen on Volatile Organic Acid Dissimilation

The free energy change which accompanies the production of molecular hydrogen from propionate and butyrate is positive at partial pressures of hydrogen above 10^{-4} atm, so if this is the mechanism for the dissimilation of these acids, then hydrogen should inhibit the dissimilation of these substrates by the enrichment cultures used.

Acetate, propionate, and butyrate enrichments were incubated in Warburg flasks under 70% N_2 and 30% CO_2. At the beginning of the experiment, the volatile organic acid corresponding to each enrichment was added anaerobically to give a final substrate concentration of 5 to 10 mM, and carboxy-labeled ^{14}C volatile organic acid were added to the pool. Liquid samples were removed at the beginning of the experiment, and at hourly intervals. After 2 hours incubation the gas phase was changed to 70% H_2 and 30% CO_2, and incubation was continued for 2 additional hours. The specific activity of the substrate was then determined. In each case, the specific activity remained constant throughout the experiment ($\pm$ 4%) indicating that no substrate was produced during the experiments. Therefore, changes in substrate levels could be interpreted as substrate utilization by the enrichments. Figures 44 to 46 show that acetate dissimilation was slightly inhibited by molecular hydrogen, while propionate dissimilation and butyrate dissimilation were completely shut off. The propionate data represents a corroboration of evidence reported by Smith and Shuba (23).

Rates of Methanogenesis in the Absence and Presence of Added Molecular Hydrogen

Studies in the lab of van den Berg (25) indicated that when enriching with acetate, the ability to utilize hydrogen as a methanogenic substrate diminished as the original inoculum was washed out. This indicates that hydrogen is not an intermediate in acetate dissimilation, for otherwise there would have been an enrichment for hydrogen oxidizing methanogens during enrichment for acetate utilizers. To determine the ability of the enrichments in the present study to utilize hydrogen, the methanogenic rates in the absence and presence of hydrogen were determined and compared. Three fifty ml samples from each of the enrichment cultures were added anaerobically to Warburg vessels with 70% N_2 and 30% CO_2 atmospheres, and gas production from each was measured hourly. After 6 hours the atmospheres were replaced with 70% H_2 and 30% CO_2, and gas uptake was measured every 10 min. for 1 hour (Table 7). Gas samples taken at the end of this hour always contained within 8% of the methane expected from hydrogen uptake by the stoichiometry of the reduction of carbon dioxide with hydrogen. To establish that the hydrogen uptake

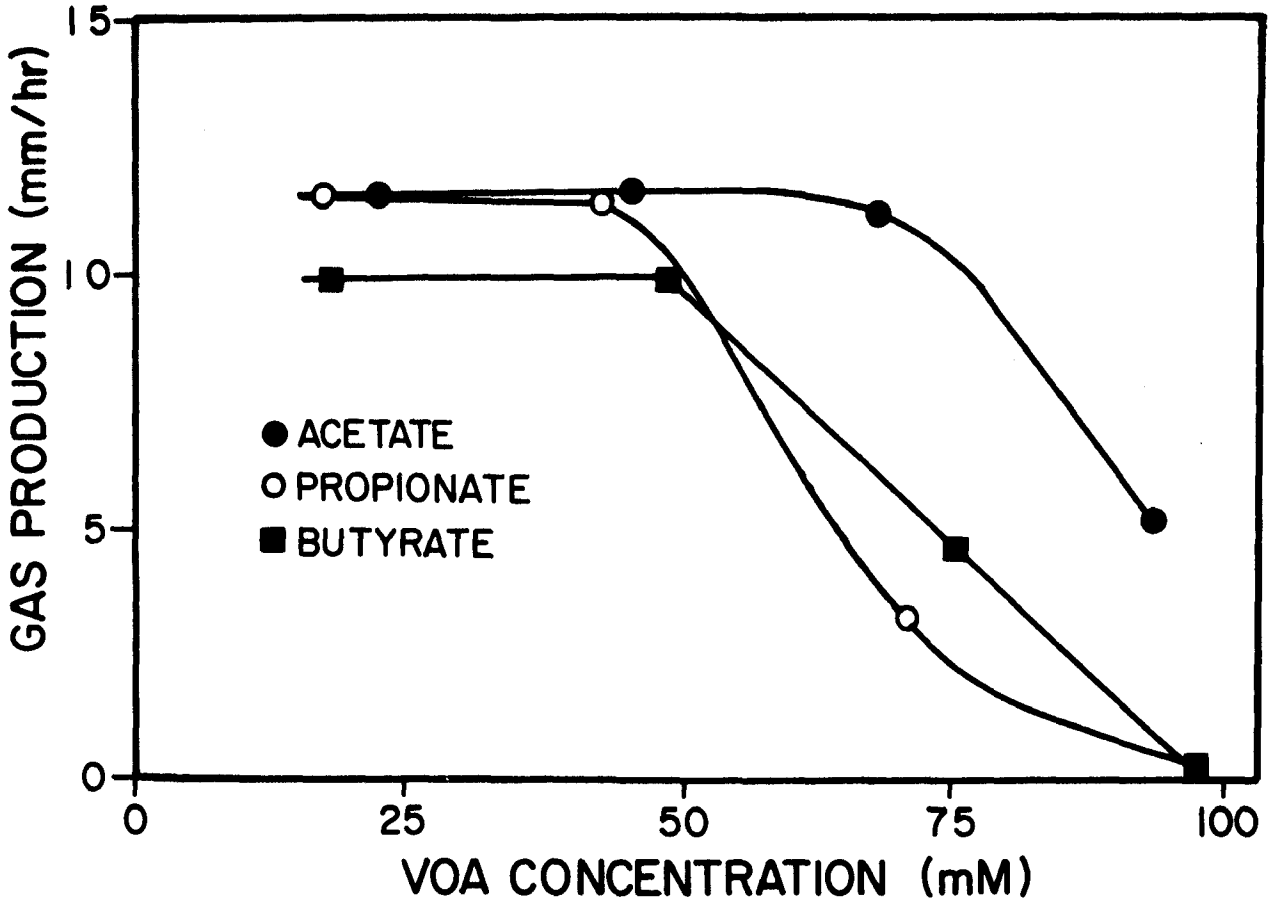

Figure 43. Inhibition of gas production by methanogenic enrichments maintained at pH 6.9 to 7.0. Acetate enrichments with added acetate, propionate enrichments with added propionate, and butyrate enrichments with added butyrate.

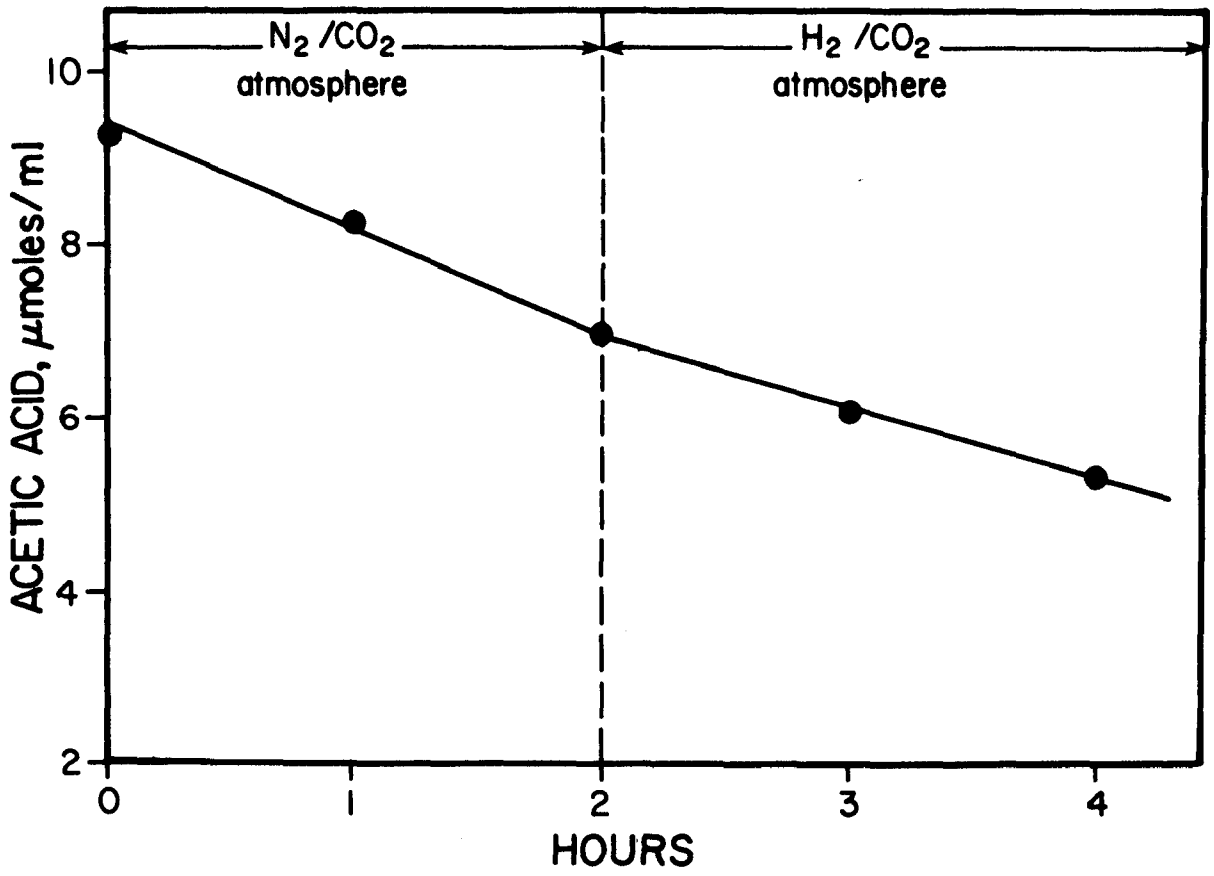

Figure 44. Degradation of acetic acid in the absence and presence of added molecular hydrogen.

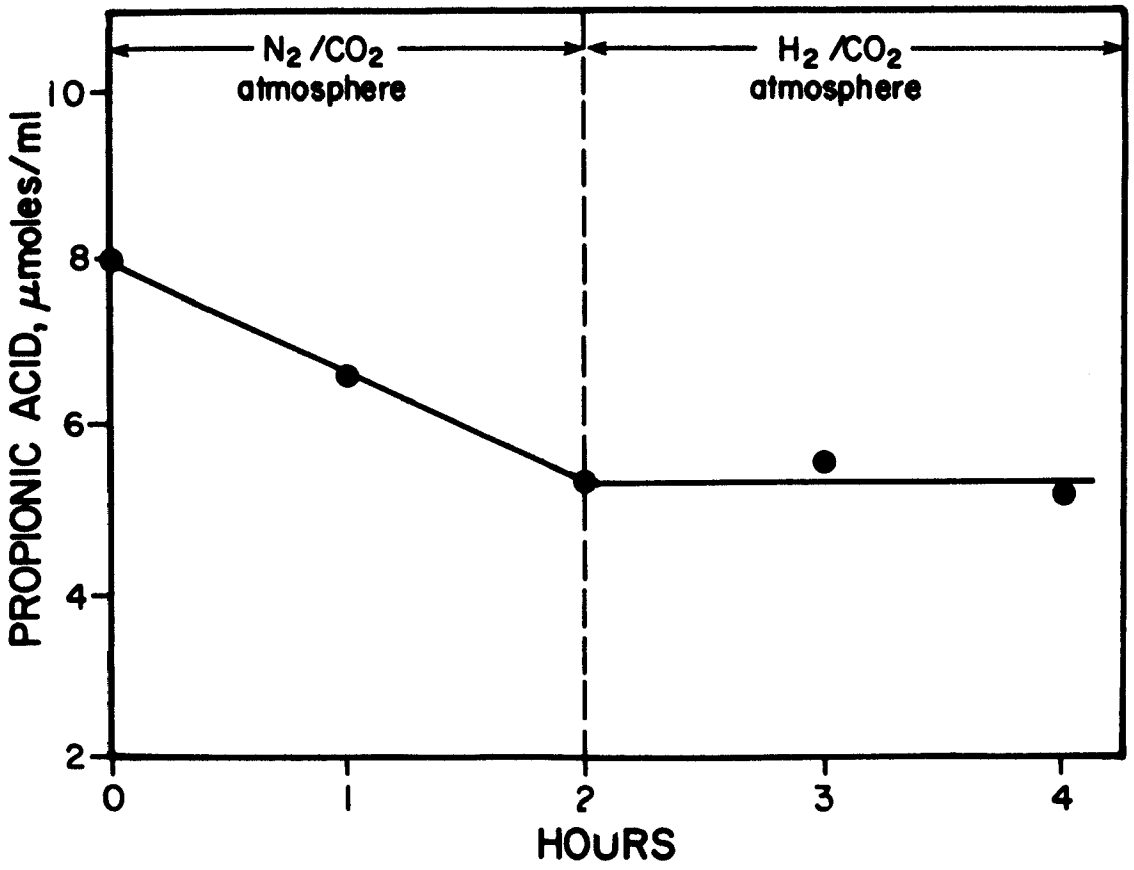

Figure 45. Degradation of propionic acid in the absence and presence of added molecular hydrogen.

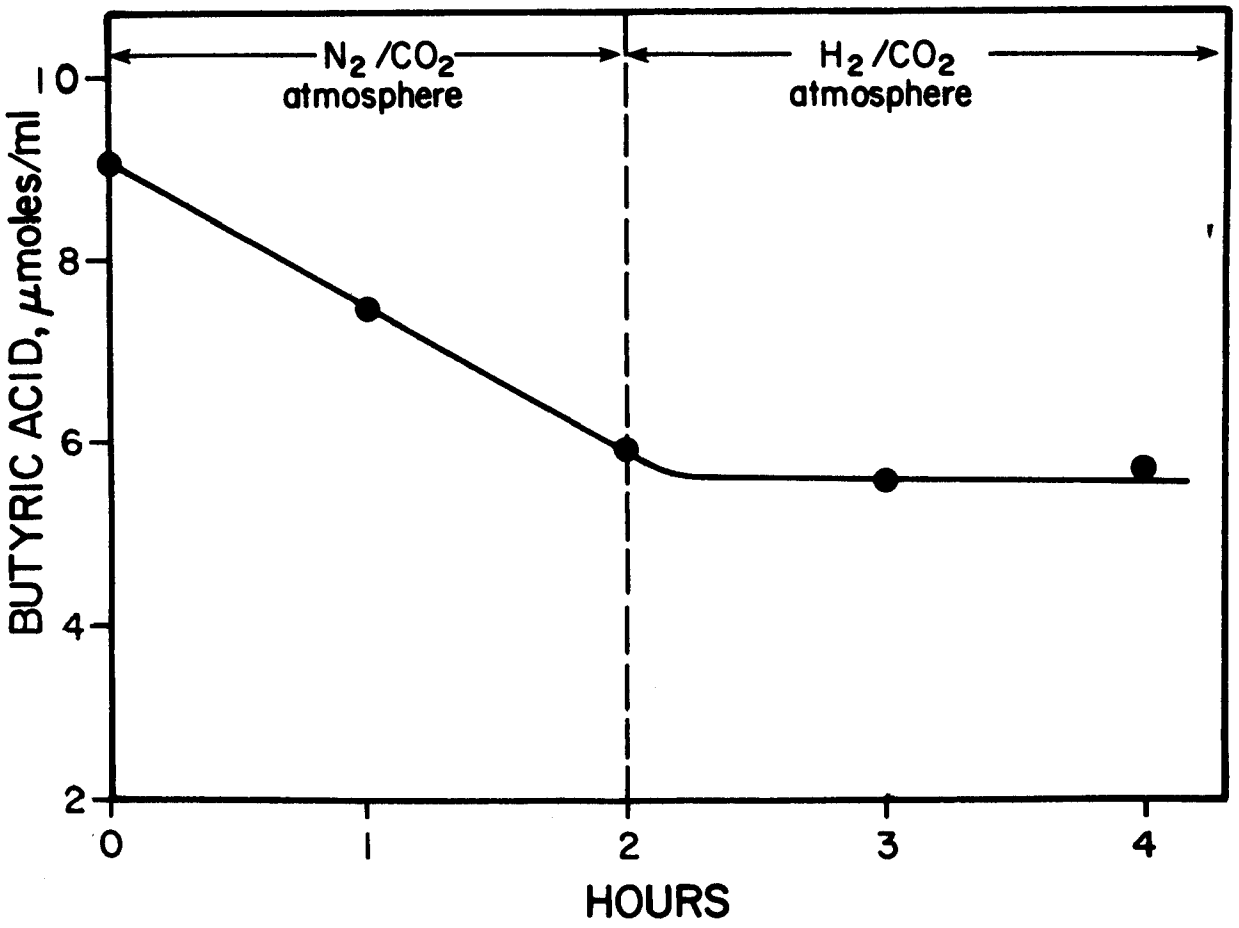

Figure 46. Degradation of butyric acid in the absence and presence of added molecular hydrogen.

resulted in carbon dioxide reduction, 50 ml aliquots of acetate enrichment were incubated in two Warburg vessels with atmospheres of 70% H_2 and 30% CO_2. Labeled sodium bicarbonate was added, and flasks were incubated for 1 hour at 30° C while monitoring gas uptake manometrically. At the end of the incubation period, 10 ml of 1 M NaOH solution were added slowly with a syringe. The NaOH solution caused the carbon dioxide to be absorbed by the liquid in the flasks. The 10 ml volume approximately replaced the volume of gas absorbed, so the total volume of liquid plus gas remained approximately the same. Ten ml of gas was then removed for quantitation of the radioactivity. Based on the specific activity of the bicarbonate and the amount of hydrogen utilized, the amount of labeled methane was calculated (assuming that all hydrogen utilized resulted in carbon dioxide reduction). In the duplicate flasks, 93% and 96% of the expected radioactivity was found in the gas samples. This demonstrates that nearly all of the hydrogen absorbed in these experiments was used for carbon dioxide reduction.

The high rates of hydrogen oxidation exhibited by propionate and butyrate enrichments demonstrate that enriching for these substrates also enriches for hydrogen oxidizing methanogens, suggesting that hydrogen may be an intermediate in the dissimilation of these substrates.

TABLE 7. METHANE PRODUCTION AND HYDROGEN UTILIZATION BY ENRICHMENT

Enrichment	Gas Production from VOA (mm/hr); N_2/CO_2 atm.	H_2 Utilization (mm/hr); H_2/CO_2 atm	Ratio of H_2 Utilization to gas production from VOA
Acetate	16.6 (±1.2)	39.2 (±2.5)	2.4 (± 0.23)
Propionate	18.9 (±1.9)	88.8 (±11.8)	4.7 (±0.61)
Butyrate	15.3 (±1.0)	82.8 (±4.8)	5.4 (±0.31)

MPN Analysis for Methanogenic Bacteria

The results of the previous experiment were corroborated by MPN analysis. Serial ten-fold dilutions of each enrichment were made using liquid media with 70% N_2 and 30% CO_2, and from these dilutions were inoculated in triplicate into roll tubes having a gas phase of 70% H_2 and 30% CO_2. The tubes were incubated at 30° C for 3 weeks, and then checked for development of a vacuum. Negative pressure development plus the presence of greater than 10% methane constituted a positive test for the presence of methanogens. Numbers of methanogens in the enrichments were calculated as follows: 1.1×10^7 per ml in the acetate enrichment, 4.4×10^8 per ml in the propionate enrichment, and 4.2×10^8 per ml in the butyrate enrichment.

Hydrogen Stripping Experiments

Hydrogen is generally not detectable in gases produced from healthy anaerobic digestors. Hydrogen was not produced by the enrichments used in this study, presumably because of the rapid oxidation of hydrogen by methanogenic bacteria present. Attempts were therefore made to remove dissolved hydrogen from enrichments before it could be utilized in methanogenesis.

A 500 ml gas scrubber was gased out with oxygen-free 100% CO_2, and 500 ml of enrichment culture was added. To prevent a decrease in pH during equilibration with 100% CO_2, 6 g $NaHCO_3$ was added. The pH, after equilibrium with 100% carbon dioxide, was between 6.8 and 6.9. Enrichments were maintained at room temperature under 100% CO_2. Carbon dioxide was then vigorously bubbled through the enrichments and the effluent gas was collected. Carbon dioxide was absorbed using the alkaline gas collector. Collections were made at various sparging rates, and the alkali insoluble gases analyzed for methane and hydrogen. The procedure was repeated on samples from each enrichment. In the case of the propionate and butyrate enrichments, feed to the enrichments was withheld 24 hours prior to its use in a sparging experiment, resulting in the substrate being dissimilated. Detectable volatile organic acids were not present. At the beginning of an experiment the desired substrate was added. This was done to limit acetate dissimilation during the experiments in which propionate and butyrate dissimilation were examined.

Figure 47 shows gases produced by the propionate enrichment when only acetate was present as substrate. Even at sparging rates approaching 1 l/min, little hydrogen was produced. Acetate enrichments dissimilating acetate gave similar results. Figure 48 shows that a different aliquot of the same propionate enrichment used in Figure 47 produced large amounts of hydrogen when propionate was present as substrate. The decrease in methane production at high sparge rates can be accounted for by the loss of methane which would have been produced from the hydrogen which had been stripped from solution. Figure 49 shows gases produced by the butyrate enrichment dissimilating butyrate, and, as with propionate dissimilation, large amounts of hydrogen were produced at high sparging rates. The decrease in the methane production at high sparge rates can again be accounted for by the loss of methane which would have been produced from the hydrogen which had been stripped from solution.

To determine the quantity of methane and hydrogen remaining in the alkaline solution of the collector, 100% nitrogen was added to the collector through the septum after collected gas had been removed at the end of a sparging experiment. The nitrogen gas was then shaken in the collector with the alkaline solution for 1 hour and removed for analysis. The gas that had remained in solution was calculated according to Henry's law and was found to represent less than 0.5% of the methane and less than 0.5% of the hydrogen produced during the sparging experiment. This was deemed insignificant, and routine analysis included only undissolved gases.

Thirty liters from each tank of 100% carbon dioxide used for sparging experiments were absorbed using NaOH solution in the gas collector. No hydrogen and no methane was detected in the alkali insoluble gas.

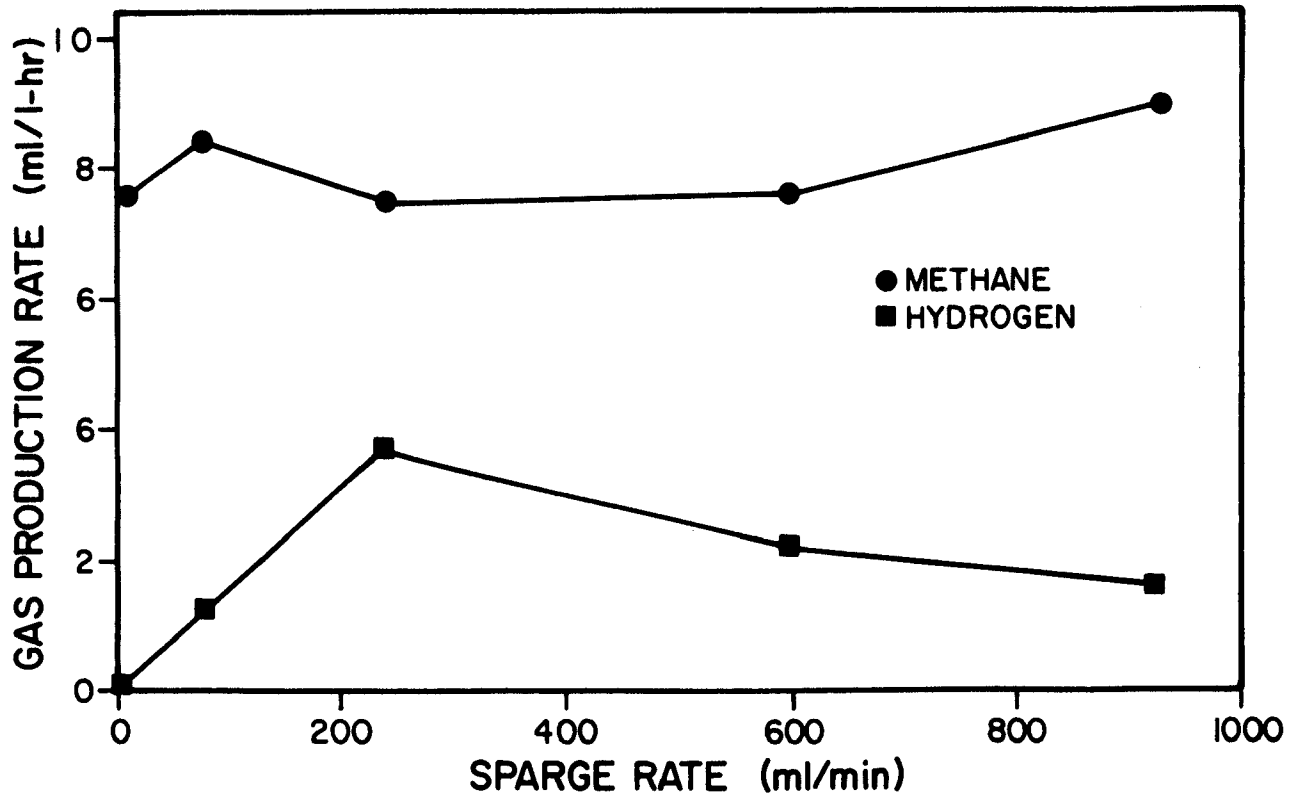

Figure 47. Gas production obtained from a propionate enrichment with acetate as the substrate, when flushed with 100 percent CO_2.

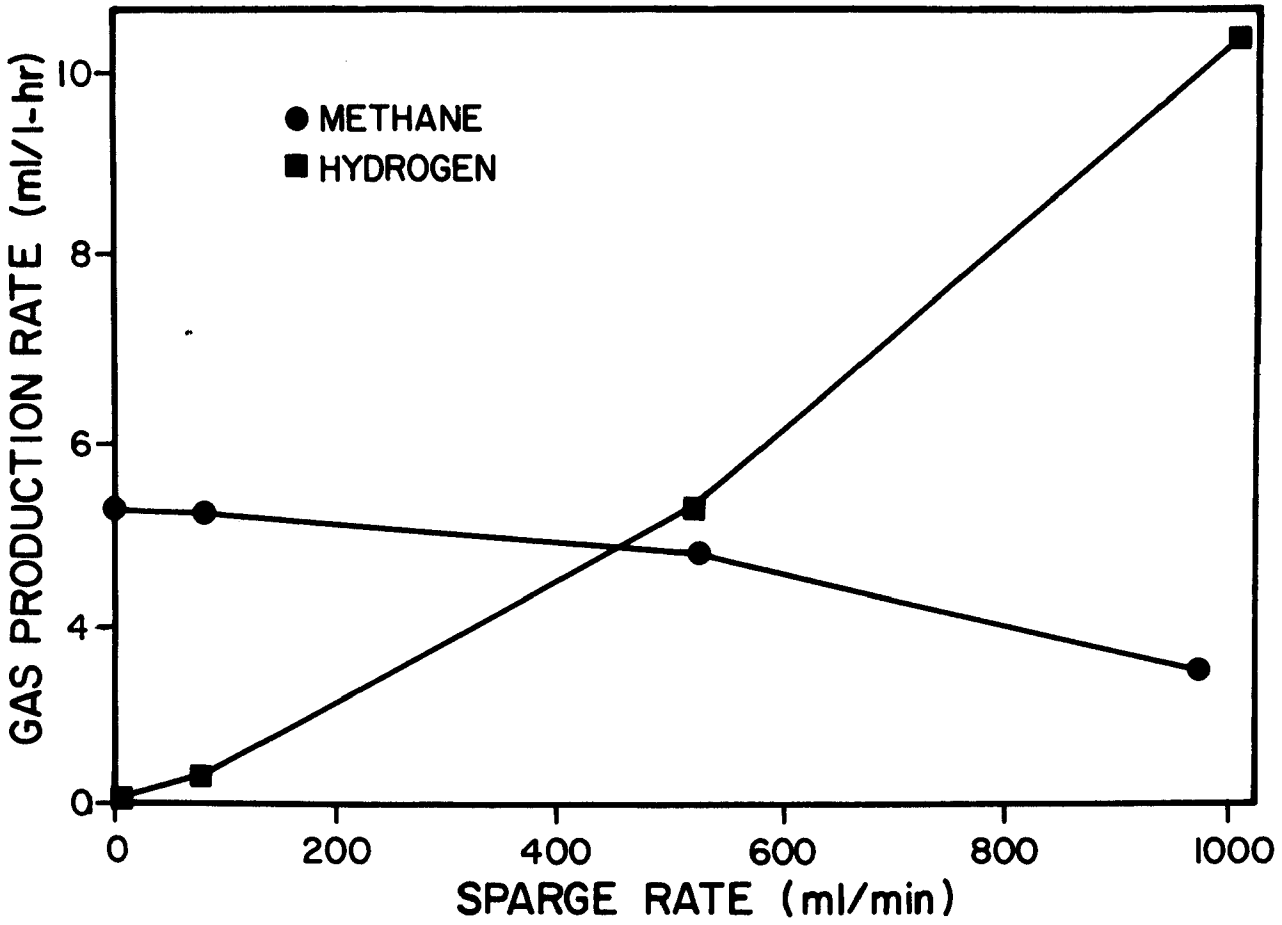

Figure 48. Gas production obtained from a propionate enrichment with propionate as substrate when flushed with 100 percent CO_2.

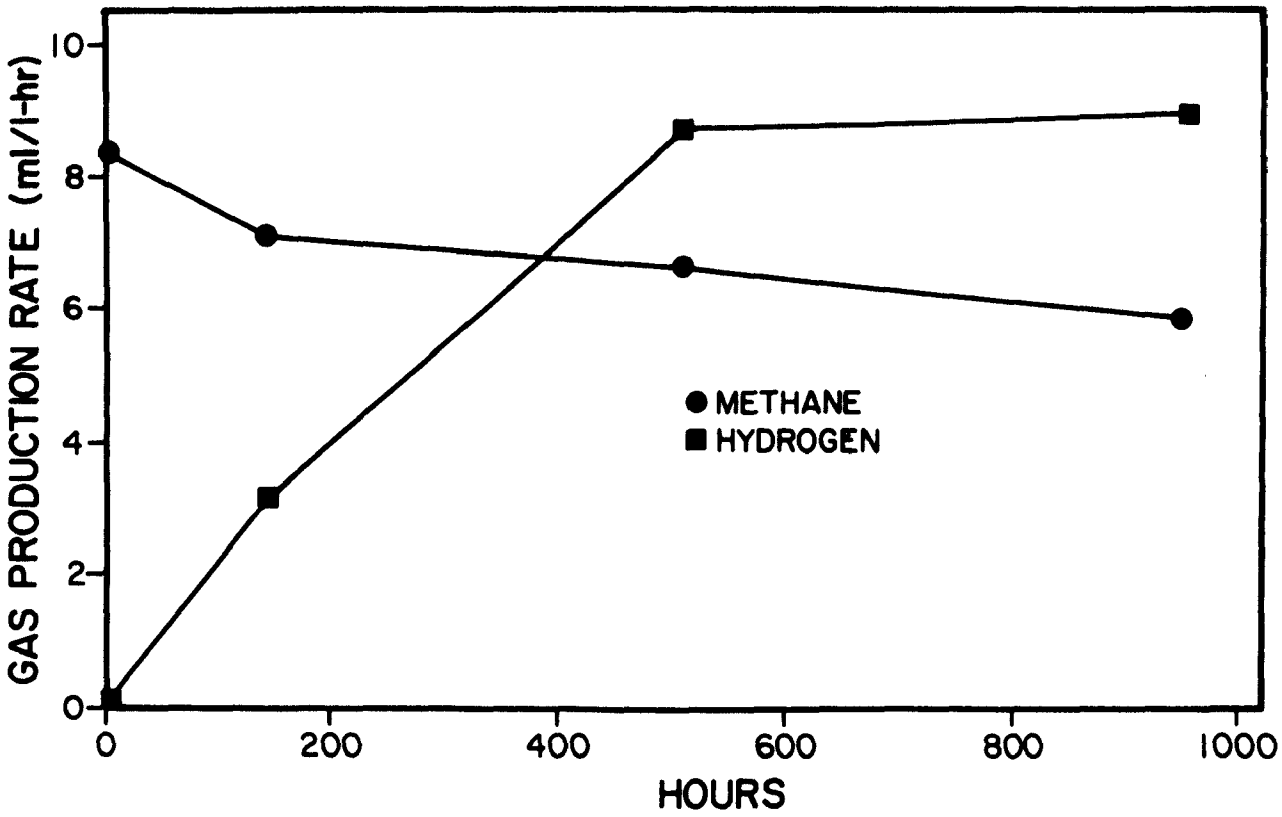

Figure 49. Gas production obtained from a butyrate enrichment with butyrate as the substrate when flushed with 100% CO_2.

Sludge from a conventional laboratory scale anaerobic digestor was also sparged, as well as sludge diluted 1:1 with sludge supernatent. The supernatent was prepared by adding sludge to a gased out flask and cooling in an ice-water bath to halt gas production which might stir up sediment. Sludge was allowed to settle for 50 min. and the supernatent was siphoned off into the gas scrubber. It was then warmed to 35° C, and an equal volume of sludge was added from the digestor. As with the enrichment sparging experiments, 6 g of $NaHCO_3$ was added to maintain pH at 6.8 to 6.9. Sludge sparging experiments were performed at 35° C and always began exactly 1 hour after the daily feeding of the digesters. Figure 50 shows the results with sludge, and Figure 51 shows the results with sludge diluted with an equal volume of sludge supernatant.

The relative small amount of hydrogen removed from the sludge may be due to high levels of solids present. Bacteria adhering to the solids may accomplish interspecies hydrogen transfer in the confines of microenvironments, preventing the removal of hydrogen by sparging.

Pure Culture Isolations

Serial ten-fold dilutions were made from each enrichment using liquid media with a nitrogen and carbon dioxide gas phase. Solid media with hydrogen and carbon dioxide gas phase and solid media with nitrogen and carbon dioxide gas phase with 25 mM VOA (corresponding to the enrichment) were inoculated from these dilutions and incubated 3 weeks at 30° C.

Slowgrowing colonies picked from the solid media, with a nitrogen and carbon dioxide gas phase, produced no colonies on attempted subculturing in identical media. Colonies picked from solid media with hydrogen and carbon dioxide gas yielded 16 methanogenic isolates after subculturing. These were isolated from original dilutions of 10^{-7} or greater. None of the isolates were able to utilize propionate or butyrate when inoculated into media with either nitrogen and carbon dioxide, or hydrogen and carbon dioxide in the gas phase.

Methanogenic propionate and butyrate enrichments were shown to have the ability to rapidly oxidize hydrogen. This allows the production of hydrogen in enrichments without its accumulation, an important consideration since thermodynamic calculations indicate that hydrogen accumulation could inhibit hydrogen production from propionate or butyrate. This possibility was confirmed by demonstrating inhibition of propionate and butyrate dissimilation in the presence of added hydrogen.

Under normal conditions the hydrogen produced in methanogenic enrichments is rapidly oxidized by the large numbers of methanogenic bacteria present. In these experiments hydrogen was removed before it could be utilized, by rapidly flushing the enrichments with carbon dioxide. Effluent gas, containing mostly CO_2, was greatly reduced in volume by absorbing the CO_2 with NaOH for ease of analysis. Using this technique, propionate and butyrate degrading enrichments were shown to produce large amounts of hydrogen.

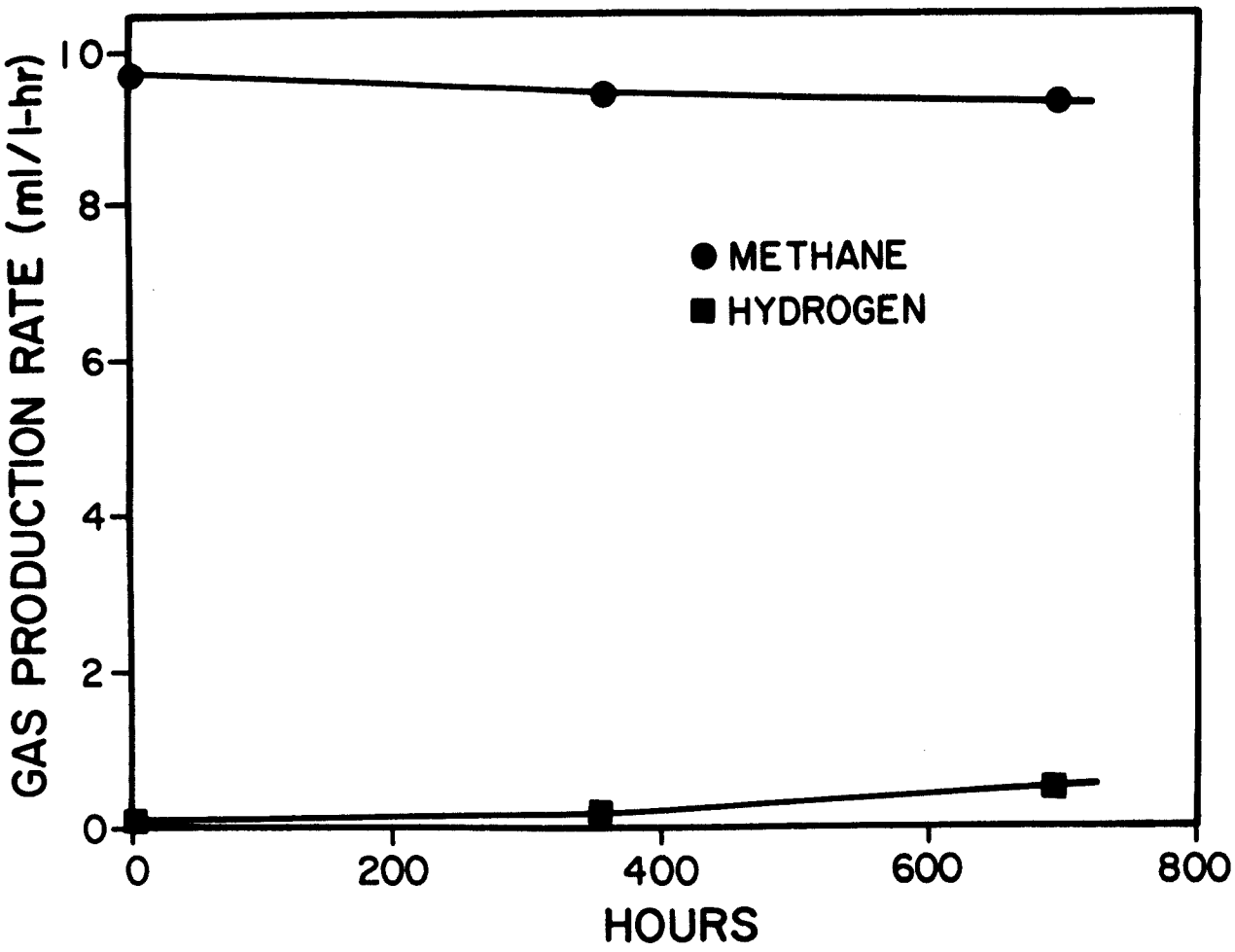

Figure 50. Gas produced from digesting sludge when flushed with 100% CO_2.

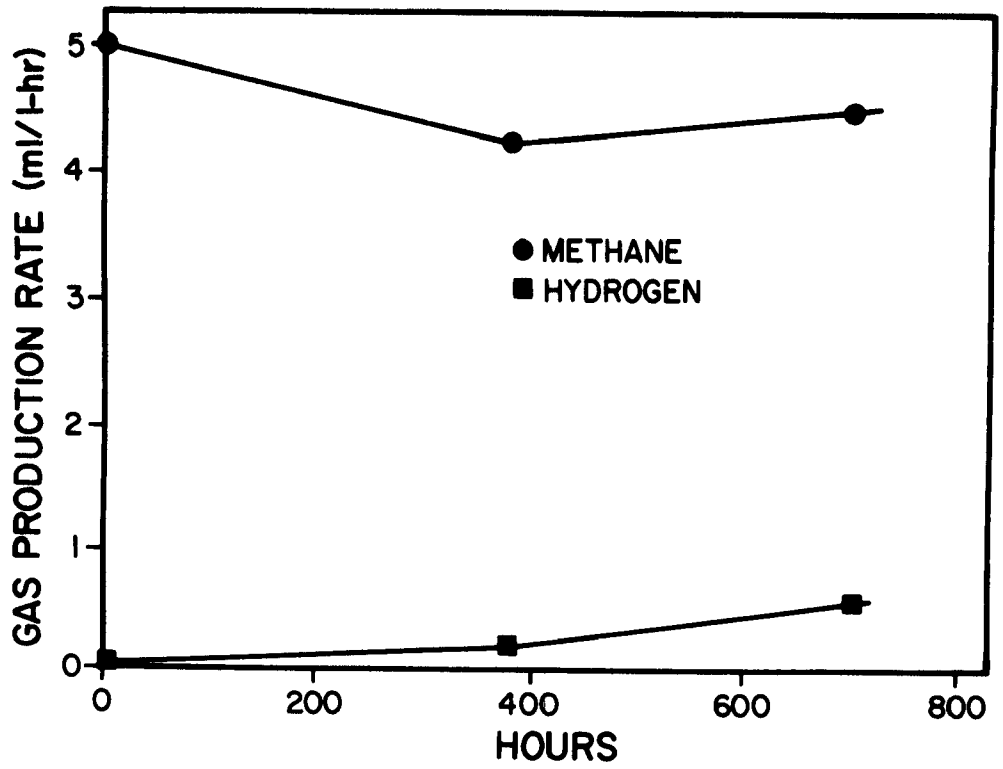

Figure 51. Gas produced from digesting sludge diluted with sludge supernatent when flushed with 100% CO_2.

In summary, the evidence that molecular hydrogen is an extracellular intermediate in the methanogenic dissimilation of propionate and butyrate is: 1) Enrichments are able to produce large amounts of hydrogen from these substrates. 2) Hydrogen inhibits propionate and butyrate dissimilation. 3) Hydrogen can be utilized by these enrichments in large amounts. 4) Enriching cultures with propionate or butyrate at the same time enriches for large numbers of hydrogen oxidizing methanogenic bacteria which cannot themselves utilize these substrates.

Models for methanogenic propionate and butyrate degradation based on the organisms known as Methanobacterium propionicum and Methanobacterium suboxydans are inconsistant with the hydrogen production demonstrated by propionate and butyrate enrichments.

A model for propionate and butyrate dissimilation involving interspecies hydrogen transfer is consistent with these results: Enrichments are able to produce large amounts of hydrogen from these substrates. Hydrogen is rapidly oxidized by methanogens present in the enrichments, so that the partial pressure of hydrogen is maintained at a level low enough to allow exergonic hydrogen production from propionate and butyrate. Only when hydrogen is rapidly removed from solution by sparging can significant quantities be detected. Artifically increased hydrogen concentrations halt propionate and butyrate dissimilation. Finally, in enriching cultures with propionate or butyrate, one at the same time enriches for hydrogen oxidizing methanogens, which cannot themselves utilize these substrates.

It is concluded that methane production from propionate and butyrate involves a hydrogenogenic and a methanogenic microflora.

ACETATE AND PROPIONATE FEED RATE EXPERIMENTS

It is generally accepted that short chain volatile acids are major intermediates in the formation of methane during sludge digestion. Work previously presented in this report further confirms this fact, quantitating the contribution of acetate, propionate, and butyrate to methane formation, and showing the interplay of hydrogen in this process. The significance of these acids and their balance in the process suggest that the pool size and turnover of these acids may limit the reaction rate under steady state conditions. If this were true an increase in the rate of formation of the acids would result in a continued increase in the pool size. If this were not the case, an increase in the rate of formation of the acids would result in an initial increase in pool size followed by the establishment of a new stable pool size, and it should also be possible to quantitatively determine the capacity of the existing microflora to metabolize additional substrate. Experiments were conducted to evaluate these questions, for acetate and propionate, using the methods previously described.

The digesting sludge used in these experiments was obtained from a seven liter laboratory digester being maintained on domestic waste with a retention time of 20 days. The digesting sludge was obtained from the laboratory

digester 2.5 hours after feeding. The rate of methane formation in control flasks was 0.017 μmoles/ml/min at 90 minutes and 0.017 μmole/ml/min at 380 minutes.

Results for acetate are shown in Figure 52. New pool sizes were established at pumping rates below 0.011 μmoles/ml/min. There results show that the sludge microflora had a capacity to metabolize acetate at a rate approximately twice the rate at which acetate was in fact being metabolized under steady state conditions 2.5 hours after feeding. The rate of methane formation from acetate, therefore, was limited by the rate of acetate formation. The sludge had a substantial ability to metabolize additional acetate under steady state conditons.

Results for propionate are shown in Figure 53. The capacity of the micro-flora to metabolize additional propionate was less than its capacity to metabolize additional acetate, as would be expected from their relative rates of metabolism in the system. New pool sizes were established at pumping rates below 0.0013 μmoles/ml/min. The results show that, as with acetate, the sludge microflora had a capacity to metabolize propionate at a rate approximately 40% faster than propionate was being metabolized under steady state conditons, assuming that 30% of the methane formed was formed through propionate. The rate of methane formation from propionate was limited by the rate of propionate formation. The sludge had a substantial ability to metabolize additional propionate under steady state conditions.

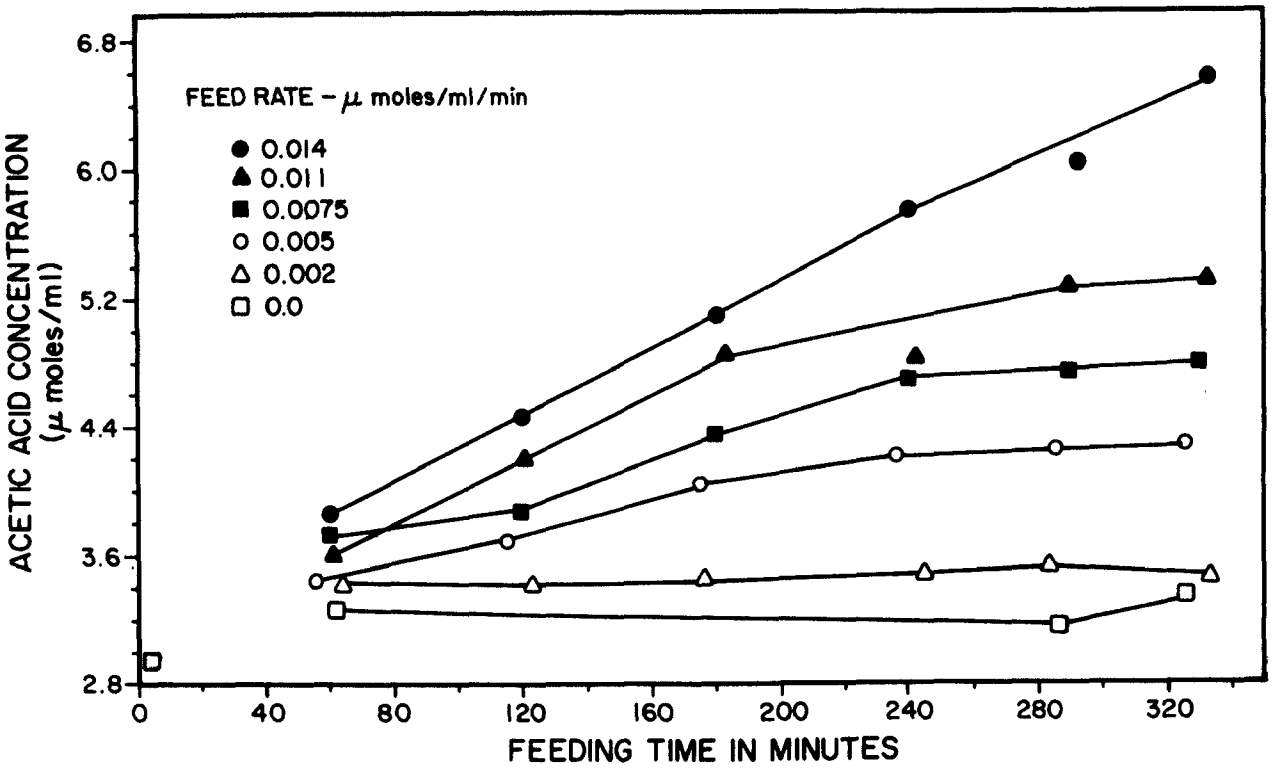

Figure 52. Changes in concentration, with time, of acetic acid in digesting sludge fed acetic acid at feed rates from 0.002 μmoles/ml/min to 0.014 μmoles/ml/min.

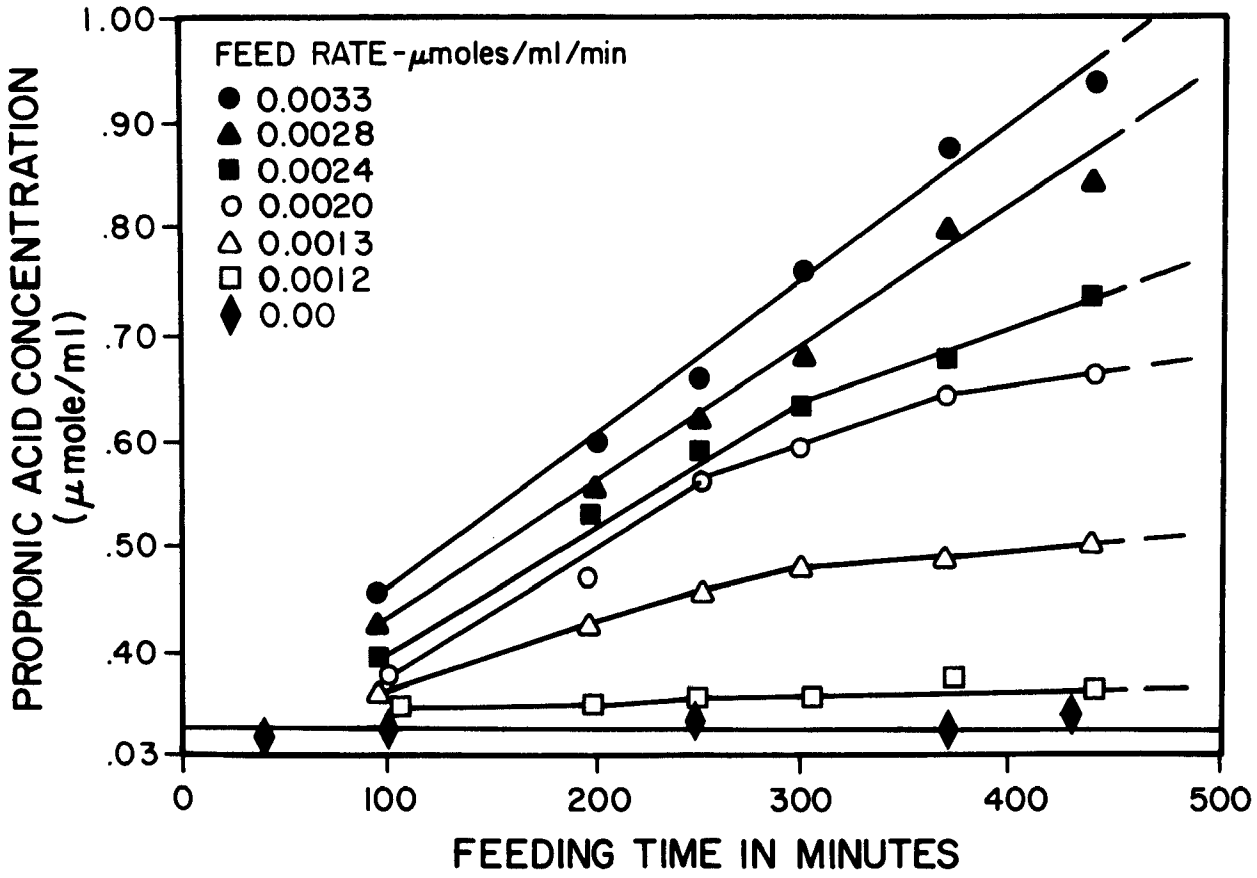

Figure 53. Changes in concentration, with time, of propionic acid in digesting sludge fed propionic acid at feed rates from 0.0012 μmoles/ml/min to 0.0033 μmoles/ml/min.

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APPENDIX A

MATERIALS AND METHODS

ISOLATION TECHNIQUES

The equipment used included the following: A copper column consisting of an 80 X 5 cm glass tube packed with fine copper turnings and containing a 400-watt immersion heating element. The outlet from the column was connected by rubber tubing to 10 cm (4 inch), 20 gauge syringe needles. The tubing preceding the needles was packed with 5.1 cm (2 inches) of sterile cotton.

A syringe pipetting assembly with a 5 ml capacity and 2 ml capacity automatic pipetting syringes was fitted with 3.8 cm (1.5 inch) number 19 needles having Huber points.

Reinforced 150 X 16 mm culture tubes were used. The culture tubes were sealed with number 0 rubber stoppers.

A 19 X 25 cm press was used to hold the stoppers in place during autoclaving. Media were prepared in a water bath adjusted to 45° C $\pm$ 1° C.

The final percentage composition of the basic medium was NaCl, 0.1; NH_4Cl, 0.05; $CaCl_2$, 0.005; $NaHCO_3$, 0.5; $MgCl_2 \cdot 6H_2O$, 0.005; KCl, 0.005; $(NH_4)_6Mo_7O_{24}$. $4H_2O$, 0.001; $CoCl_2 \cdot 6H_2O$, 0.001; resazurin, 0.001; L-cysteine hydrochloride hydrate, 0.05; $Na_2S \cdot 9H_2O$, 0.05; agar, 1.5; rumen fluid, 30. The 30 percent rumen fluid was added as a source of nutrients. Substitution of 30 percent sludge supernatant, 0.3 percent yeast extract, 0.3 percent beef extract, or 0.3 percent trypticase soy broth for the 30 percent rumen fluid was not satisfactory.

Medium was prepared containing all ingredients except rumen fluid, cysteine, $Na_2S \cdot 9H_2O$, and $NaHCO_3$. The medium was boiled to drive off dissolved oxygen. The atmosphere above the medium was maintained free of oxygen by flushing with a gas mixture of 70 percent hydrogen and 30 percent carbon dioxide. The gas mixture was freed of oxygen by passing it through a hot copper column. Rumen fluid, cysteine, and $NaHCO_3$ were added after the medium had been cooled to 45° C. The medium was distributed in 5 ml quantities into the culture tubes using the automatic pipetting assembly. The gas in culture tubes was freed of oxygen by flushing with the gas mixture prior to the introduction of the medium. The culture tubes were maintained free of oxygen and sealed with rubber stoppers. This was done by passing the gas mixture through a syringe needle inserted into the culture tube. The gas mixture flowed through the needle at a rate sufficient to keep the tube anaerobic. The

rubber stopper was firmly seated as the syringe needle was removed. After the stoppers were inserted the culture tubes were placed in test tube racks, clamped in the press, and sterilized at 120° C for 15 minutes. After cooling at 45° C in a water bath, 0.05 ml of 5 percent $Na_2S \cdot 9H_2O$ was injected aseptically through the stoppers using an automatic pipetting syringe. Dilution blanks were prepared in the same way and had the same composition as the medium except that agar was omitted.

The substrates in the medium described above were either hydrogen or an organic substrate added to give a final concentration of 0.3 percent. The sodium sulfide was added immediately before the medium was inoculated. A 5 percent $NaS \cdot 9H_2O$ solution was prepared under helium and injected into each tube immediately prior to inoculation.

Samples to be inoculated were suspended in dilution blanks and then injected into culture tubes containing growth medium and agar. Appropriate dilutions were rolled in an ice bath until the agar gelled. Inoculated tubes were then incubated at the proper temperature.

DETERMINATION OF TURNOVER RATES

Procedures used were similar to those described by Smith and Mah (22) or as described below.

Fifty ml aliquots of sludge from a laboratory digester were placed in Warburg respirometers. The respirometers were 100 ml nominal volume and equipped with sampling ports closed with rubber serum caps. The sludge was kept anoxic during transfer by flushing the digester and respirometers with 70% N_2 : 30% CO_2, and filling the transfer pipet with the same gas mixture. The gas mixture was freed of traces of oxygen by passage through a column packed with hot, freshly reduced copper turnings. The sludge in the respirometers was equilibrated with the gas mixture for 15 minutes static, and 15 minutes at a shake rate of 105 strokes/min. The respirometers were then brought to atmospheric pressure and closed, and an elapsed time clock started. Clock time was recorded for each addition to the respirometers of each sample taken from them. One-half ml aliquots of radioisotope solutions were added with a syringe through the serum caps. Radioisotope was added to two respirometers, two respirometers were used for measurement of methane production, and one respirometer was used for fatty acid pool measurements. The respirometer was used for fatty acid pool measurements. The respirometers were maintained at 35° C and a shake rate of 105/min, in a Warburg water bath. Manometer excursions were recorded over the experimental period, and then gas samples were taken for methane analysis. Samples were taken from the respirometers with added labelled compounds at approximately 10 min. intervals over a 60 min. period. Syringes in metal pipetting holders, fitted with 19 gauge needles, were used to remove 1 ml samples from the respirometers, in the case of acetate and propionate. The syringes were flushed with 70% N_2 : 30% CO_2 prior to sampling. Samples were taken through the rubber serum caps and injected into test tubes containing 0.5 ml of carrier acid in H_3PO_4, and pre-chilled in an ice bath. Samples were kept in the ice bath until the end of the experiment, and then they were placed in a freezer at -20° C until analyzed.

In the case of butyrate, where the pool size was very low, the procedure was changed. Butyrate samples were taken by removing the respirometer from the Warburg bath, removing the serum cap, and pipetting out two 20 ml aliquots. In this case a large number of respirometers was used. Anoxic conditions were maintained with a flow of 70% N_2 : 30% O_2 during the operation. The 20 ml aliquots were run into duplicate 50 ml, teflon-lined, stainless steel centrifuge tubes. The centrifuge tubes were in an ice bath, and contained 2.5 ml 0.25N NaOH each. Immediately after the experiment, they were centrifuged for 15 min. at 37,000 x g and 0° C. The supernatent liquid was then decanted and stored at -20° C overnight. The duplicate butyrate samples were combined and 25 ml of each combined sample were pipetted into 500 ml round-bottom flasks. Two glass boiling beads and 2.00 ml of carrier plus internal standard solution were added to each. The samples were then dried on a rotary evaporator, under reduced pressure at 60-70° C. The residue was dissolved in 2.00 ml 20% H_3PO_4 prior to analysis. Resulting samples analyzed for internal standard (n-valerate) concentration showed a maximum variation of less than 4%; hence the measured radioactivity in the samples was not corrected for concentration differences.

Organic acid pool samples were taken and chilled in the same manner as samples for ^{14}C-acetate and ^{14}C-propionate samples (carrier and acid were omitted from the test tubes), and the samples were frozen immediately after chilling 2-5 min. Response was used to calculate concentrations of methane in samples, based on response when known amounts were injected.

Organic acid pools were measured by centrifuging sludge samples for 10 min. at 27,000 x g and 0° C, acidifying 2.00 ml of the supernatent liquid with 0.20 ml of 20% H_3PO_4, and injecting 2 µl of the acidified solutions into the chromatograph. A Hamilton 10 µl syringe, equippped with a Chaney constant-volume adapter, was used to inject the solutions. Organic acids were separated on a 2.4 m x 3.2 mm glass column, packed with 15% SP-1000 (Supelco Inc. Bellefonte, Pa.) and 2% H_3PO_4 on 70/80 mesh anakrom A (Analobs Inc, Handen, Conn.) Injection flash heater temperature was 145° C, column oven temperature was 130° C, and nitrogen flow rate was 25 ml/min. Acetate and propionate were measured at 3 x 10^{-10} amps/mv sensitivity and butyrate at 3 x 10^{-11} amps/mv. All other conditions were the same as for methane analysis. Standards for each acid were injected immediately after the samples.

When radioactive fractions were to be trapped, the SP-1000 column was replaced with a 1.5 m x 6 mm glass column packed with 15% FFAP (Varian-Aerograph, Walmit Creek, Ca.) and 2% H_3PO_4 on 70/80 mesh anakrom A. Samples were centrifuged at 27,000 x g and 0° C for 15 min., and 8 µl of the supernatant were injected into the chromatograph. Column oven temperature was 105° C and nitrogen flow rate was 50 ml/min. A 16 : 1 stream splitter was connected to the exit end of the column, with the minor portion of flow going to the detector, and the major portion to a heated outlet tube. The outlet tube was 2 mm diameter stainless steel, heated to 160° C by means of a surrounding jacket. The outlet end of the tube extended 10 mm beyond the end of the jacket. Fractions were trapped by observing the chart trace and connecting a trap to the end of the outlet tube when the desired organic acid was emerging. Traps were 100 mm x 6 mm teflon tubes, packed to a depth of 45 mm with

Porapak Q, and closed at the bottom with a plug of glass wool and a perforated silicone rubber plug. Traps were connected to the outlet tube by inserting the free end of the tube through the rubber plug. Trapped samples were immediately washed off the Porapak with ethanol which had been dried over anhydrous Na_2SO_4. The trap was connected to a short piece of 2 mm diameter stainless steel tubing inserted through a rubber stopper. The stopper was also penetrated with a 19 gauge hypodermic needle connected to a aspirator pump. The stopper was inserted into a scintillation vial, the aspirator turned on, and 5 ml ethanol passed through the trap into the vial. Ten ml of scintillation fluid (0.4% PPO, 0.01% POPOP in toluene) were added to the ethanol solution in the vial, and the mixture counted in an Packard Tri-Carb scintillation counter. Counting efficiency of the system was 70%. Samples were counted from 3 to 10 minutes, depending on activity observed.

RADIOISOTOPE, CARRIER AND STANDARD SOLUTIONS

2-^{14}C-labelled acids were supplied at 99% radiopurity, as sodium salts. Acetate was obtained from Calbiochem, Los Angeles, Ca., at a specific activity of 22.5 µci/µmole. Propionate was obtained from ICN, City of Industry, Ca., at a specific activity of 52 µci/µmole. Butyrate was obtained from Volk Radiochemical, Burbank, Ca., at a specific activity of 10 µci/µmole. Solutions containing 170 µci/ml 2-^{14}C-acetate, 57 µci/ml 2-^{14}C-propionate and 1.3 µci/ml 2-^{14}C-butyrate were prepared, using boiled distilled water and sealed under nitrogen gas with serum caps. Solutions were prepared on the same day they were used.

Carrier for acetate and propionate samples was 15 µmole/ml propionic acid in 1.8 N H_3PO_4 solution. Carrier (and internal standard) solution for butyrate was 4.3. µmole/ml n-butyric acid and 8.5 µmole/ml n-valeric acid, in water.

Standards for organic acid analyses were prepared fresh, immediately prior to analysis. Stock solutions of approximately 100 µmole/ml acetic, propionic, and butyric acids (titrated against standard NaOH) were diluted to give standards containing 9.89 µmole/ml acetic acid, 3.92 µmole/ml propionic acid and 0.432 and 0.216 µmole/ml butyric acid. Error approximations, based only on tolerances of glassware used in all dilutions and titrations, and on probable error in weighing primary standard (potassium acid phthalate), indicate final concentrations in standards should be accurate to better than $\pm$ 2%.

ANALYSIS AND RADIOISOTOPE ASSAYS

Duplicate 1 ml gas samples from the respirometers were analyzed for methane using a Packard model 7829 gas chromatograph. Samples were taken and injected into the chromatograph with a Hamilton 1 ml gas-tight syringe. Methane was separated on a 1.5 m x 6 mm glass column, packed with 50/80 mesh Porapak Q (Waters Assoc., Framingham, Mass.) and detected with a flame ionization detector. The column oven temperature was 105° C, carrier (N_2) flow rate was 20 ml/min., hydrogen flow rate was 20 ml/min. and air flow rate was 500 ml/min. A sensitivity of 1 x 10-7 amps for 1 mv output was used. The

output signal was displayed on a 28 cm (11 in.), 1mv recorder, equipped with a Disc integrator.

DIGESTOR EXPERIMENTS

Eight-liter glass bottles served as laboratory scale anaerobic digestors. These digestors were totally sealed from the atmosphere. They had inlet and outlet ports and were stirred with internal 5 mm teflon tubes which were driven by low speed motors connected to low speed high torque motors. The digesters were fed raw sludge which was stored in a frozen state. Gas production was measured by water displacement.

HYDROGEN TRAPPING EXPERIMENTS

Anaerobic Digestor

An 8-l glass bottle served as a laboratory scale anaerobic sludge digestor. Anaerobically digested domestic wastewater sludge obtained from the Gainesville, Florida, Wastewater Treatment Plant served as an inoculum. The sludge was collected and handled in containers previously gassed out with oxygen-free nitrogen gas. The laboratory scale digestor was maintained at 35° C in a water bath, and was mixed 15 min. every hr. Each day 250 ml of sludge were removed and 250 ml of raw sludge added; digestor liquid volume was maintained at 7 l. Raw sludge was obtained from the Gainesville plant, blended in a Waring blender for 5 min., and frozen in polypropylene bottles for storage. Gas production, measured by displacement of water, increased slightly over the first week of incubation and then stabilized at 4.2 to 4.8 l per day. Evolved gas was composed of 63 to 72% methane.

Gases

All gases were obtained from the Matheson Gas Company. Gas mixtures for routine digestor, manometry, and culture work were Purified grade. Traces of oxygen were removed by passing gases over freshly reduced hot copper filings. Carbon dioxide for sparging experiments (Anaerobe grade) was passed through two gas scrubbers containing 500 ml of 0.018 M titanous chloride solution which removed traces of oxygen (22), and saturated the gas with water. Gases for chromatography were Purified grade. Standards used in the quantitative analysis of gases were prepared from Ultra-high purity gases. All gas mixtures were 30% carbon dioxide, with the balance either nitrogen or hydrogen.

Determination of Volatile Organic Acids

Volatile organic acids were quantitatively determined using a Packard 800 Series gas chromatograph with a 1.8 m by 2 mm glass column packed with 10% SP1000 (Supelco, Bellefonte, Pa.) and 1% phosphoric acid coated on 70/80 mesh Anakrom AW (Supelco). Temperatures were: inlet 150° C, column 122° C, detector 132° C, and outlet 148° C. The carrier gas (100% nitrogen) flow rate was 25 ml per min. A flame ionization detector was used with hydrogen

and air flow rates to the detector of 30 ml/min and 300 ml/min, respectively. The signal from the detector was connected to an Autolab Minigrator which was used for comparison to standards. Samples were prepared for analysis by mixing 0.9 ml of sample with 0.1 ml of 30% phosphoric acid, followed by centrifugation to remove cells. The injection volume was 0.4 µl.

Determination of Radioactivity in Volatile Organic Acids

VOA were separated chromatographically in a manner similar to that described above. The injection volume was increased to 4 µl and a stream splitter (ratio ca. 18:1) was inserted in the line of the column effluent, so that one part of the effluent went to the detector and eighteen parts were diverted to a port protruding through the column oven wall. Pasteur pipets were lightly packed with glass wool, and through-hole septa (Supelco, Belefonte, PA) were placed in the top. Just prior to use, the glass wool was wetted with a solution of 1 N KOH in anhydrous ethanol. The septum was inserted into the side port of the chromatograph just prior to the time the desired volatile organic acid was eluting from the column and removed when elution was complete. The acid trapped on the alkaline glass wool was washed into a scintillation vial with 10 ml of scintillation fluid (4 g 2,5-Diphenyloxazole, 0.1 g 1,4-bis-[2-(5-Phenyloxazolyl)]-benzene, and 250 ml of dry ethanol per liter of scintillation grade toluene). The amount of volatile organic acid sent to the detector was quantitated to determine by difference the amount diverted to the collector. Recovery of standard solutions of labeled volatile organic acids was greater than 90%. Samples were counted in a Beckman LS-133 Liquid Scintillation System. Counting efficiency was greater than 90%. For each sample, six replicate analyses were performed and averaged.

Manometry

All manometric experiments were performed in 100 ml Warburg flasks having a single side arm fitted with a septum. An 18 gauge needle was inserted into the septum to provide an air exit while the flasks were being gased out with oxygen-free gas. The septa were removed for introduction of 50 ml sample enrichment cultures, which were transferred in volumetric pipets under anaerobic conditions. The septa were immediately replaced, and gasing continued. After 5 min. the enrichment cultures were shaken at a rate of 70 strokes per min. for 5 min. to allow equilibration of the gas with the liquid. Needles were then removed, and the flasks were equilibrated to atmospheric pressure and sealed. Experiments were then started. To change atmospheres in the vessels during an experiment, needles were re-inserted through the septa and the flasks gased out with the second gas mixture.

For volatile organic acid analysis, liquid samples of 0.9 ml were removed using syringes previously gased out with oxygen-free gas.

Collection of Sparge Gases

Effluent gas from sparging experiments consisted primarily of 100% carbon dioxide. Large volumes of the gas were absorbed quickly by bubbling

through excess NaOH solution in a one-liter Erlenmeyer flask sealed with a three-hole stopper. The sparge gases entered the flask through one hole, via a glass tube reaching to the bottom of the flask. As the gas bubbled up, liquid was displaced through a second hole that had another glass tube reaching to the bottom of the flask. This tube connected to a reservoir containing NaOH solution. The third hole was filled by a short piece of glass tubing sealed at the top by a septum, through which alkali insoluble gases were removed by a syringe at the end of an experiment. At very high sparge rates the flask was placed on a reciprocal shaker to increase the rate of carbon dioxide absorption. At the end of the collection period the flask was shaken 5 additional minutes to allow complete absorption of the carbon dioxide.

Quantitation of Radioactivity in Gases

A 250 ml Cary-Tolbert Ionization Chamber was used in conjunction with a Cary 401 Vibrating Reed Electrometer for quantitation of radioactivity in gases. The Rate-of-Charge method was used to determine the current through the ionization chamber with an applied voltage of 90 V. A standard curve was prepared using various amounts of $Ba^{14}CO_3$ with a specific activity of 1.16×10^3 dpm/mg (New England Nuclear, Boston). The $Ba^{14}CO_3$ was converted to gas using a carbon dioxide generator (Applied Physics Corporation, No. 3120000).

Quantitation of Gases

Gases were quantitated using a Packard 800 Series Gas Chromatograph. A 1.8 m by 2 mm glass column was packed with Carboseive B (Supelco, Bellefonte, Pa.). Temperatures were: inlet 100° C, column 70° C, detector 82° C, and outlet 96° C. Carrier gas flow rate was 30 ml/min of nitrogen. Detection was by thermal conductivity with a bridge current of 200 mA. The signal from the detector was connected to an Autolab Minigrator which was used to quantitate by comparison with prepared standards.

ACETATE AND PROPIONATE FEED RATE EXPERIMENTS

Stock 1.0 M solutions of sodium acetate and sodium propionate were prepared. Feed solutions were made by dilution of the stock solutions. All solutions were prepared with boiled distilled water which had been cooled in an atmosphere of 70% Nitrogen-30% CO_2. All solutions were flushed with the same gas mixture if exposure to air had occurred, e.g., while filling syringes, etc. The concentrations of feed solutions were measured on the gas chromatograph, and syringe pumps were individually calibrated by pumping solutions into aliquots of H_3PO_4 for 3 hours and then measuring the resulting concentrations.

Disposable 2.5 ml syringes were filled with the feed solutions and attached to a Harvard infusion pump (model 600-950). With the syringes and pump rate setting used, the delivery rate was 0.0038 ± 0.0001 ml/minute.

Incubation vessels were 125 ml respirometer vessels with sampling ports closed with rubber serum caps. Feed solutions were delivered through 21 gauge

needles inserted through the serum caps. Teflon capillary was attached to the needles to deliver the solutions below the liquid level in the flasks. Fifty ml aliquots of sludge from a 7-liter laboratory digestor (20 day detention time), were transferred, into the incubation vessels under a stream of O_2-free N_2-CO_2.

The Harvard pump was started prior to t = 0 for the experimental vessels. The teflon delivery tubes were carefully wiped immediately prior to fitting the stoppers into the incubation vessels. Zero-time for each sample was noted as the time when that flask was stoppered.

The flasks were incubated in a shaking water bath (new Brunswick model RW-150) at 35° C and 100 strokes/minute. At various time intervals, 1 ml samples were removed through the serum caps with 2 ml automatic pipetting syringes equipped with 3.8 cm 19-gauge needles. The syringe was flushed thoroughly with 70%-30% N_2-CO_2 prior to sampling. Samples were killed by injection into pre-chilled test tubes containing 0.5 ml 20% H_3PO_4 and kept in an ice bath. All samples were then frozen and kept in the freezer until analyzed, at which time they were thawed and filtered.

APPENDIX B

FERMENTATION OF RUM SLOPS

In early 1970 EPA had an interest in determining the feasibility of fermenting Puerto Rico rum slop. A problem was encountered in establishing a viable fermentation and advice was requested from this project, with the results which follow.

OBJECTIVE AND PROCEDURES

Rum slop samples obtained from Mr. Edmond Lomasney (U. S. EPA Region IV) was examined to determine its fermentability characteristics. The slop was assayed for chemical oxygen demand, ionic strength, ion composition, volatile organic content, normality, pH, and buffering capacity. Short-term fermentability was examined manometrically. Long-term fermentability was examined by feeding an established digester.

RESULTS

Slop Composition

Chemical Oxygen Demand (COD)--

The average COD for the samples in our lab was 88,800 mg/l ($\pm$ 3,100). This is a good agreement with the reported values determined in Puerto Rico. We therefore assumed the sample was representative.

Ionic Strength

Conductivity measurements on the slop indicated an ionic strength equivalent to 0.25 M NaCl. The slop is probably hypertonic for most non-halophilic bacteria. The ionic strength of digesting sewage sludge in our laboratory digesters is equivalent to 0.075 - 0.078 M NaCl. The high ionic strength may be inhibitory to a rapid fermentation.

Individual Ions Present--

The slop was assayed for individual ions by atomic absorption spectrophotometry, colorimetric assay, and the use of specific ion electrodes. The results are listed in Table B-1. Chloride is quite high.

Volatile Compounds--

Gas chromatographic analyses of the slop demonstrated the following concentrations of volatile organic acids: acetic, 14 μ mole per ml; propionic, iso-butyric, n-butyric, iso-valeric, and n-valeric, all less than 0.1 μ mole

TABLE B-1. IONS AND THEIR CONCENTRATIONS IN RUM SLOP

Ion	mg/l	Percent
Fe	76.2	0.0076
Zn	3.5	0.00035
Cu	7.5	0.00075
K	8,300	0.83
AL	25	0.0025
Ca	1,900	0.19
Mg	1,400	0.14
P	130	0.013
Cl	27,000	2.7

TABLE B-2. pH CHANGES OF RUM SLOPS DUE TO ADDITION OF SLUDGE

Manometer Number	Ml Sludge	Ml Slop	Initial pH
1	25	0	7.2
2	25	1	7.2
3	25	2	7.1
4	25	5	6.9
5	25	10	6.6
6	25	15	6.4
7	25	20	6.2
8	10	25	5.2
9	5	25	5.0
10	0	25	4.9

per ml. The ethanol concentration was 0.54 μ moles per ml. (0.02 percent). These substances, at these concentrations, should have no adverse effect on the fermentation.

Normality, pH and Buffering Capacity--

The pH of the slop sample was 4.9. Titration of a sample indicated a normality of 0.05. This indicated that the slop could lower the pH of sludge and inhibit the fermentation. Various combinations of sludge and slop were mixed together to determine the pH change (see Table B-2).

Short-term Manometric Measurements

The short-term fermentability of the slop was determined by adding slop to digesting sludge and measuring the resulting fermentation over a five day period. Conditions and results are shown in Tables B-3 and B-4.

Warburg flasks were used as fermentation vessels. Anaerobic techniques were employed in all phases of the experiment. Vessels and slop were gased with 70 percent N_2: 30 percent CO_2 prior to adding sludge.

After adding sludge to the manometer vessels, they were closed and shaken in a 35 degree C water bath. The excursions resulting from gas production were followed and at the intervals listed below gas samples were analyzed for methane by gas chromatography.

Results Obtained the First Day of Incubation--

After 150 minutes of shaking, the gas produced was analyzed for methane. In Table B-3, methane production for the first day represents the percent methane produced in relation to that produced by the controls.

Second Day Results--

The manometers were opened and the gas produced for 18 hours was allowed to escape through a 27 gauge needle. On the second day the manometers were closed and shaken for 150 minutes. Methane in each vessel was measured; the gas was released; and vessels were closed again and shaken for 230 minutes. The gas produced was again assayed for methane. The methane production reported in Table B-3 is the difference between the levels found at 150 minutes and the levels found after 230 minutes of additional shaking.

Fifth Day Results--

The mixtures of sludge and slop were then allowed to incubate without shaking for an additional 65 hours. Gas produced during this interval was allowed to escape through a 27 gauge needle. The manometers were then closed and the gas was analyzed for methane. After 330 minutes of shaking, the gas produced in each manometer vessel was again assayed for methane. The methane levels reported in Table B-3 are differences between the levels found at the time of closing the vessels and 330 minutes of shaking. After 5 days of incubation, final pH, percent hydrogen, and final VOA pools were checked. These properties are given in Table B-4.

TABLE B-3. GAS PRODUCTION FROM DIGESTING SLUDGE DILUTED WITH RUM SLOPS

Manometer Number	Ml Sludge	Ml Slop	Methane produced as percent of the controls		
			Day 1	Day 2	Day 5
1 (Control)	25	0	100	100	100
2	25	1	106	110	0
3	25	2	130	114	100
4	25	5	118	122	115
5	25	10	96	153	133
6	25	15	84	69	0
7	25	20	55	25	4
8	10	25	0	0	0
9	5	25	0	0	0
10	0	25	0	0	0

TABLE B-4. PRODUCTS FORMED BY SLUDGE DILUTED WITH RUM SLOPS

Manometer Number	Ml Sludge	Ml Slop	Final pH	Percent H_2	VOA (μ moles per ml)					
					Acetic	Propionic	i-butyric	n-butyric	i-valeric	n-valeric
1	25	0	7.15	0.02	*	*	*	*	*	*
2	25	1	7.15	0.02	*	*	*	*	*	*
3	25	2	7.15	0.02	0.2	*	*	*	*	*
4	25	5	7.15	0.02	0.3	3.0	0.7	*	0.2	*
5	25	10	6.85	0.02	4.0	29.0	3.0	5.0	3.0	22.0
6	25	15	5.45	0.05	93.0	30.0	3.0	10.0	3.0	22.0
7	25	20	5.25	0.05	99.0	43.0	1.0	16.0	0.4	5.0
8	10	25	4.60	6.0	66.0	4.0	* *	2.0	*	0.5
9	5	25	4.70	4.0	45.0	0.8	* *	*	*	*
10	0	25	4.30	0	22.0	*	* *	*	*	*
11	25	0	7.15	0.02	*	*	*	*	*	*

* Less than 0.1 μ mole per ml.

* * Two large peaks coinciding with the iso-butyrate peak.

Long-term Feed Experiments

The long-term experiments involved feeding slop to an anaerobic digester. A 4 liter digester, containing 3.5 liters of digesting sewage sludge, was used. The digester had been fed 175 ml of raw domestic sludge per day prior to changing to slop feed. Gas production ranged between 5.8 to 6.2 ml/min for the first two hours after feeding during a two week period prior to the change over. During this same period the total gas produced during the first five hours after feeding ranged from 1.4 - 1.6 liters with a composition of 69 - 74 percent methane. Total gas produced for twenty-four hours after feeding was 5.8 - 6.2 liters. The following parameters were measured in this part of the study: a) rate of gas evolution for the first five hours after feeding; b) percent methane and the average μ moles methane per minute per ml liquid for the first five hours; c) total gas produced in twenty-four hours; d) pH twenty-four hours after feeding; e) ionic strength twenty-four hours after feeding; f) VOA concentrations five and/or twenty-four hours after feeding.

Because of the high ionic strength it was decided to dilute the slop with water. The dilution rates were:

Days	ml Slop	ml Water
1-5	100	100
6-11	150	50
12-16	170	60

On the seventeenth day the feed was changed to 100 ml slop and 100 ml raw sludge in an attempt to increase gas production and the percent methane.

Figures B-1 and B-2 show the increase in VOA each day 24 hours after feeding. Up to the eighth day the concentration of all VOA was less than 0.1 μ mole per ml. On the tenth day acetate, propionate and n-butyrate began to increase and continued to increase throughout the period of feeding. Iso-valerate is not included in Figure B-2 because its concentration never exceeded 1.0 μ mole per ml.

Figure B-3 shows the daily increase in ionic strength (measured by conductivity and equivalent to the molarity of NaCl). The increase in VOA pools beginning on the tenth day is correlated to an ionic strength of 0.140 M.

Figure B-4 shows the total gas produced 5 and 24 hours after feeding. Gas production for the first 5 hours was greater than, or equivalent to, the gas produced from raw sludge feed. However, total gas produced in 24 hours was always less than on raw sludge. Gas production for 24 hours did increase between 6 - 10 days. This is correlated to an increase in the amount of slop fed (see feed schedule). The general trend for total gas in 24 hours, however, is a constant decrease. Since gas production was good for the first 5 hours, the slop probably contains readily degradable substrates.

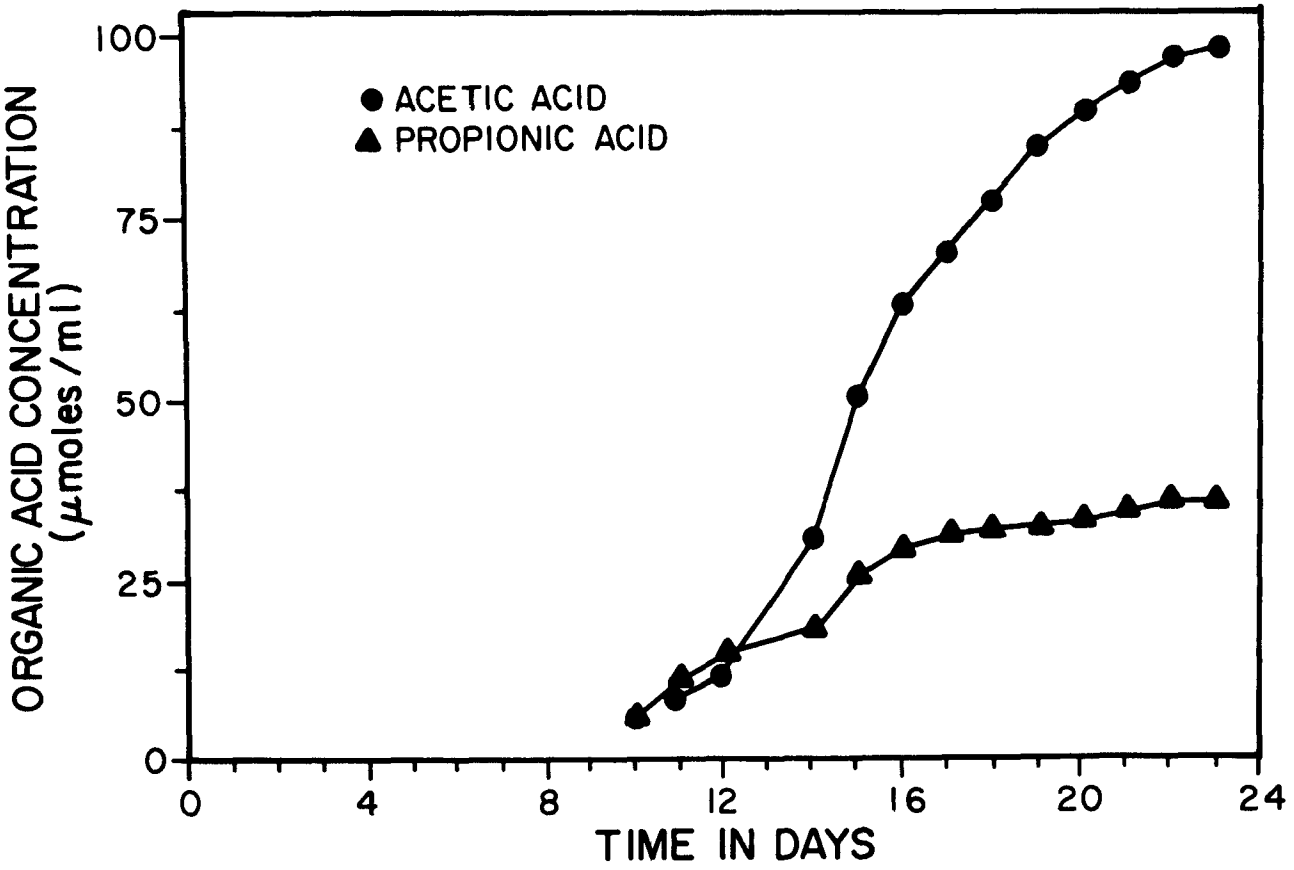

Figure B-1. Acetic acid and propionic acid concentrations each day, 24 hours after feeding.

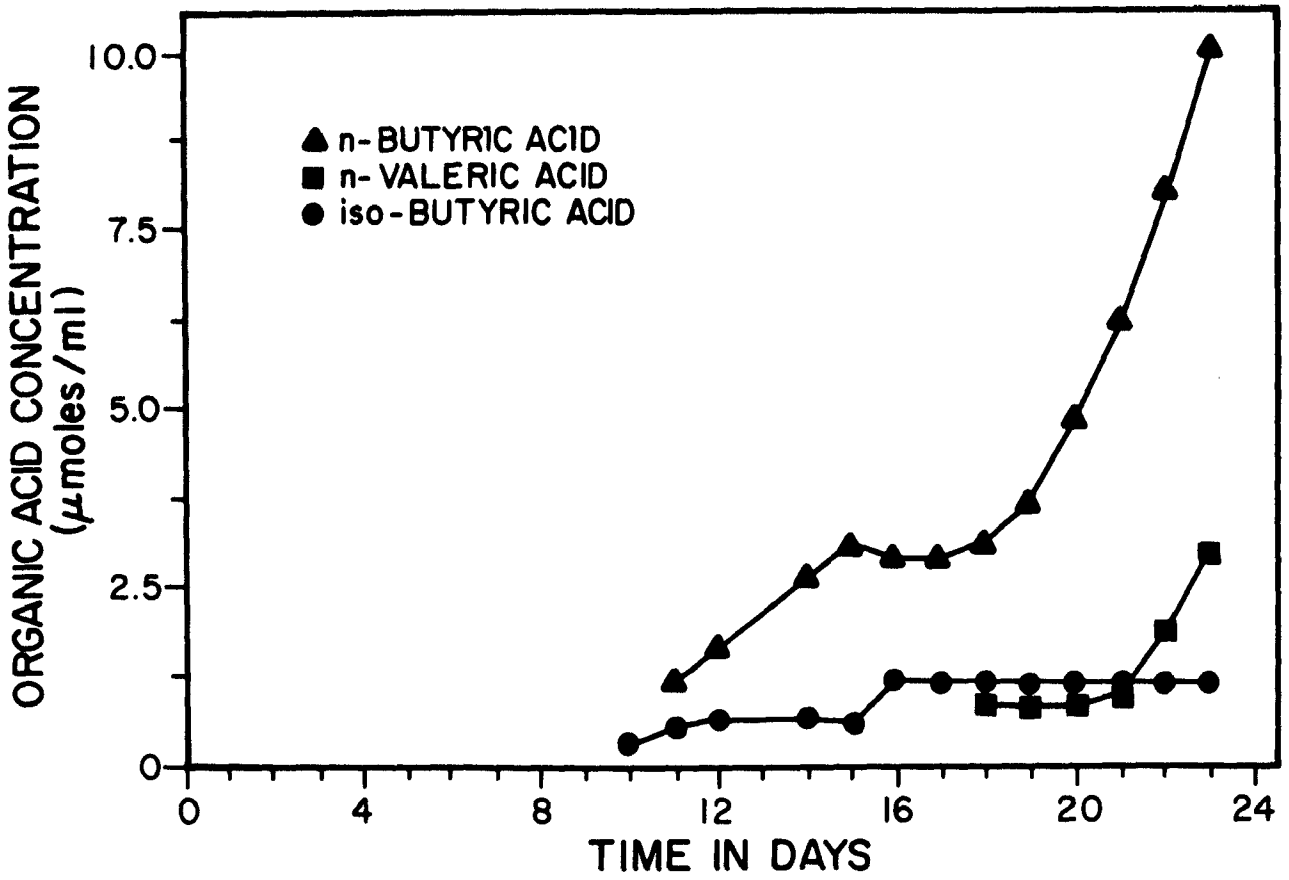

Figure B-2. n-Butyric acid, iso-butyric acid and n-valeric acid concentrations each day 24 hours after feeding.

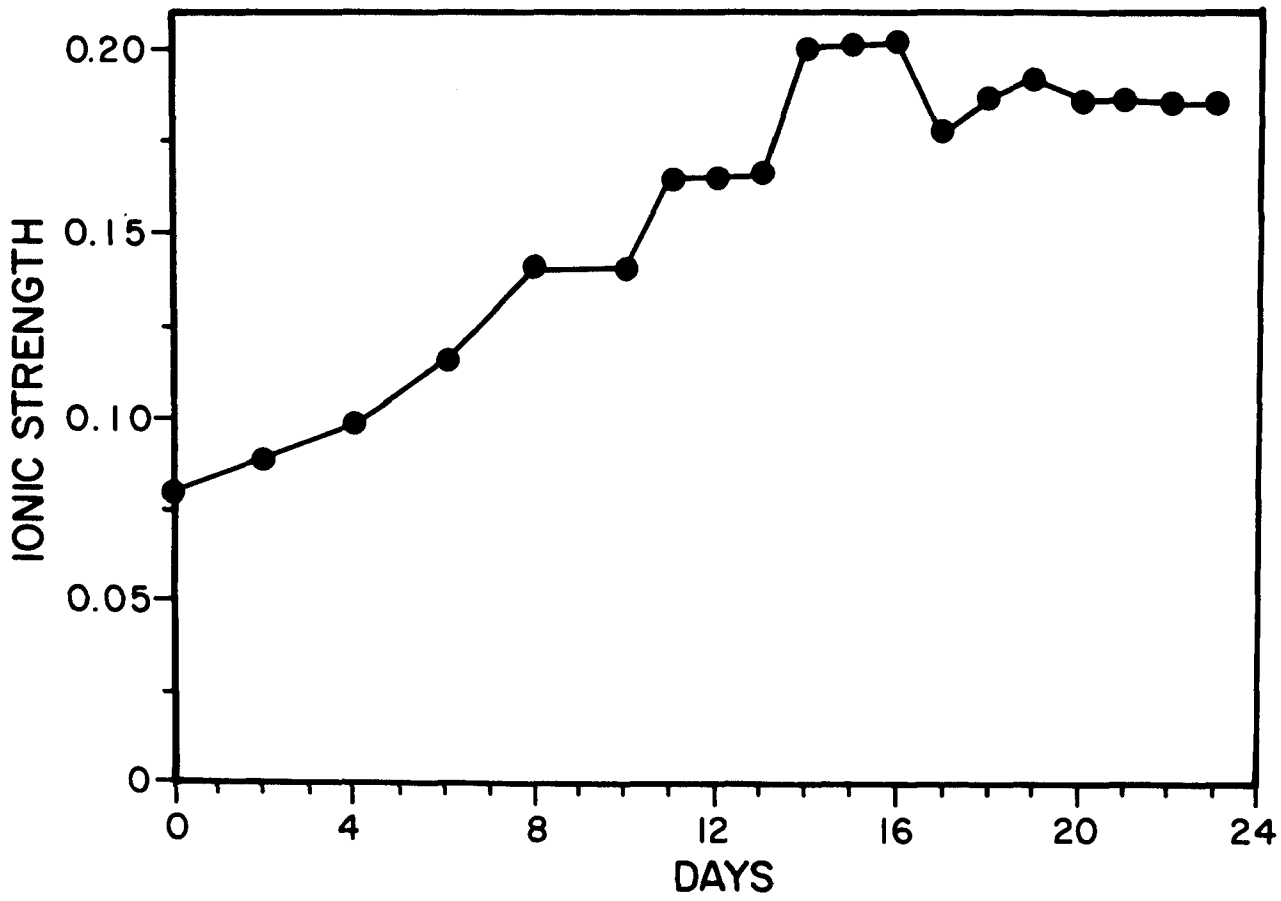

Figure B-3. Ionic strength each day, 24 hours after feeding.

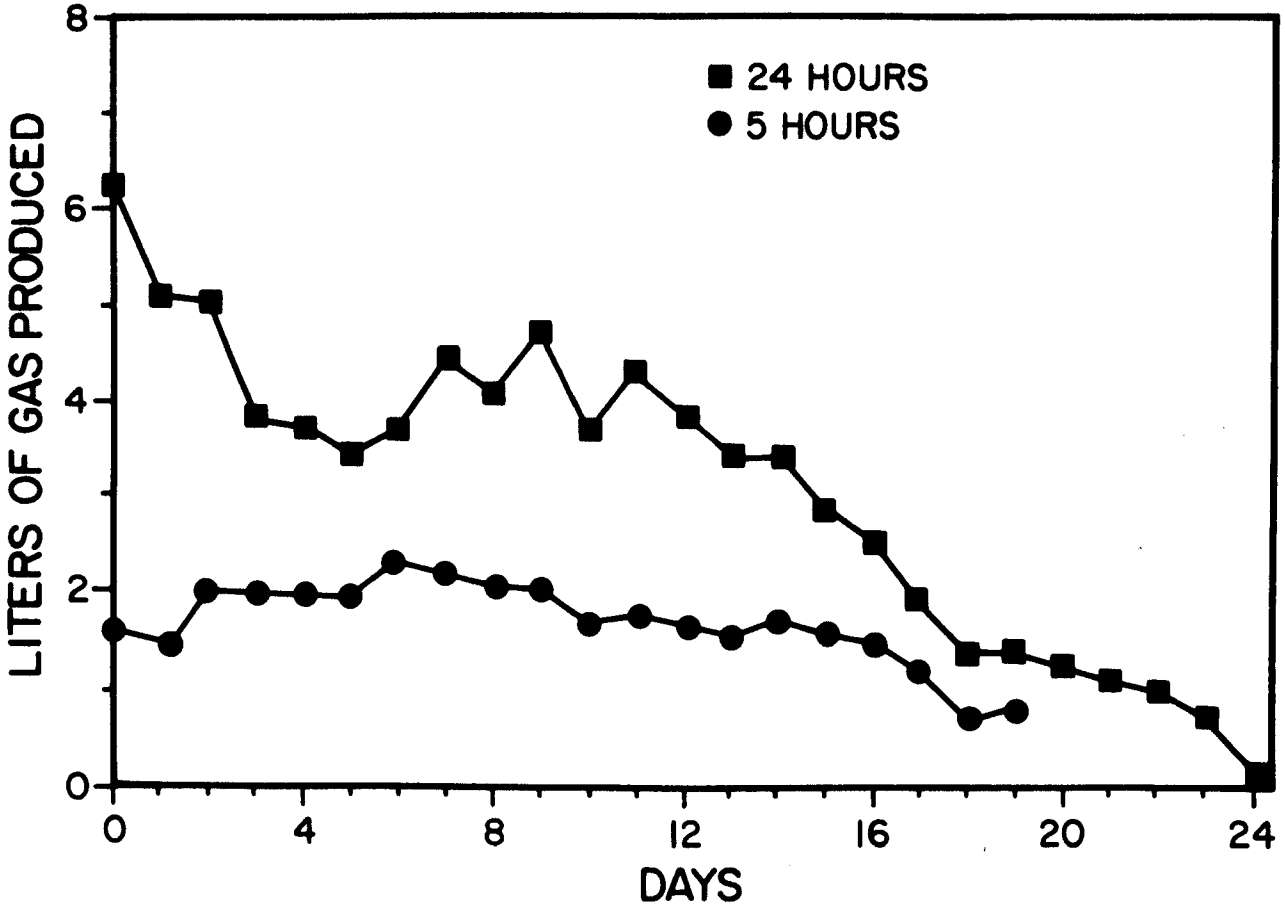

Figure B-4. Liters of gas produced each day 5 and 24 hours after feeding.

Figure B-5 illustrates the percent methane and the μ moles of methane per min per ml of liquid 5 hours after feeding. The percent methane in the gas decreases to 50 percent and remains relatively stable for 8 days when it begins to decrease. Except for an initial increase, the μ moles of methane per min per ml constantly decreases.

Assuming the slop consists mostly of readily degradable carbohydrates $(C_6H_{12}O_6)_x$, a gas phase of about 50 percent methane would be expected from the available hydrogen. This could explain the decrease in the percent methane for six days and a leveling off for the next eight days.

The pH of the digested slop did not decrease markedly as the VOA pools increased. The starting pH was 7.1. It decreased to 6.9 by the fifteenth day and went down to 6.4 by the twenty-third day.

The long term feed experiments indicated that the slop will not maintain the activity of an anaerobic digester having a microflora developed on domestic waste.

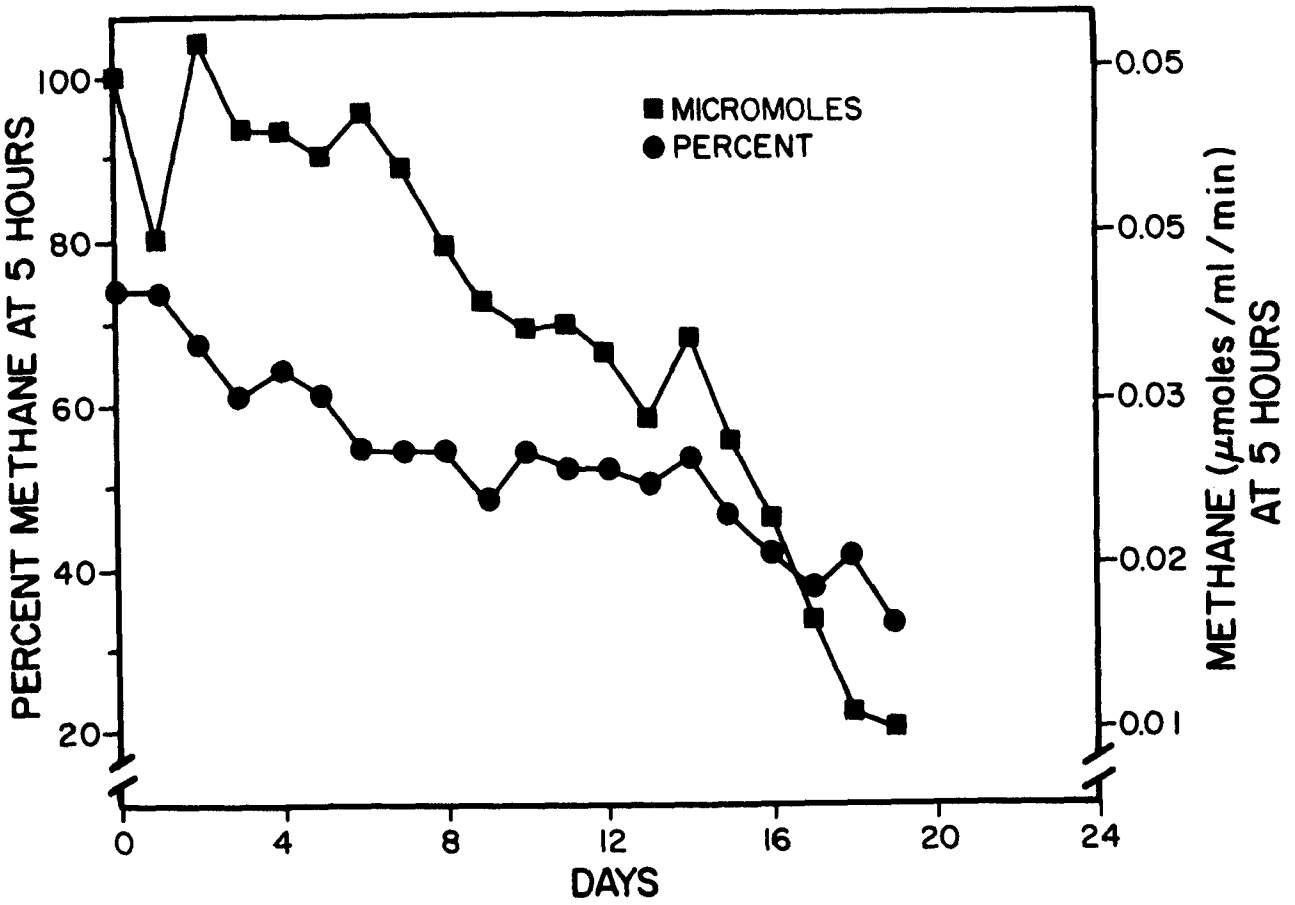

Figure B-5. Daily methane produced 5 hours after feeding.

Comments and Recommendations

The results of the study with a single sample of the rum slop suggest that some difficulty will be encountered in establishing and maintaining a fermentation of this material using a seed of sludge from a domestic digester. The data suggest that the developed ionic strength in the fermentation is a major problem, although other factors may be involved. I project that the ionic strength of undiluted fermenting slop would develop to a value exceeding 0.5 M NaCl. This is based on the fact that the slop fed was diluted, and measurements were minimal values.

Feeding diluted slop the ionic strength developed to that equivalent to 0.20 M NaCl in sixteen days, in contrast to the calculated value of 0.14 M NaCl.

It is recommended that a digester be established with a domestic sludge seed, or a marine sediment seed.

Domestic Seed--Start with 3.5 L of domestic sludge. Feed daily 125 ml H_2O, 75 ml slop and 25 ml of Metracal. Maintain for 3 weeks on this feed and then slowly increase the slop (5 ml/day), decrease the H_2O (5 ml/day), and decrease the Metracal (2 ml/day) to the desired retention time. It may be possible to maintain the fermentation on slop with no additions. This would have to be arrived at empirically.

Marine Sediment Seed--Obtain 3.5 liters of black bay mud. Obtain the mud from under decaying sea weed, if possible. Add 300 ml of slop and allow to stand until gas evolution occurs. Then start adding slop at an increasing rate. Start at 20 ml/day and increase to 200 ml/day over a period of approximately 20 days. The maximum feed rates could be arrived at empirically.

If conventional digester systems do not work, I would recommend that the feasibility of an anaerobic filter be tested.

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15. SUPPLEMENTARY NOTES

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16. ABSTRACT

Methanogenic bacteria were isolated from mesophilic anaerobic digesters. The isolates were able to utilize H_2 and CO_2 acetate, formate and methanol, but were not able to metabolize propionate and butyrate. It was shown the propionate and butyrate are not substrates for methanogenic bacteria but are converted to hydrogen, carbon dioxide and acetate by a hydrogenogenic microflora. The reactions leading to methane were quantitatively analyzed. It was shown that acetate, propionate and butyrate metabolism were inhibited by hydrogen. The formation of acetate and propionate were shown to be rate limiting in the digestion process, and that sludge digestion was not inhibited by hydrogen under conditions of excess substrate.

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17. KEY WORDS AND DOCUMENT ANALYSIS

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